POSTER SESSION I

Iron Diseases

0001

5 YEARS OF COMBINED CHELATION THERAPY: A RADICAL CHANGE IN BETA-THALASSAEMIA MAJOR PATIENT CONDITION

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Background. Transfusional iron overload in Thalassaemia major is fatal in the second decade of life unless treated appropriately. The ultimate goal of iron chelation therapy is to prevent organ damage and premature death. Combined chelation with Deferiprone (Ferriprox®) &Desferrioxamine (Desferal®) produces a synergistic iron chelating effect that is difficult to achieve with either drug alone. This approach may place all patients in negative net iron balance and lead to a significant reduction of the body iron load. *Aim.* To show which iron-induced complications may be reversible with the use of combined chelation in Thalassaemia major patients. *Methods.* 50 β-Thalassaemia major patients (TMps) aged 6-46 years, switched from Desferrioxamine monotherapy to combined chelation with oral Deferiprone (25-30 mg/kg t.i.d) and Desferrioxamine (20-50 mg/kg, 8-12h SC or IV 2-6 days/week), in a 5 year regimen, adjusted on individual needs. The following tests were routinely performed: - mean annual Ferritin based on monthly measurements by MEIA; - annual/biannual ECG and Cardiac Echo for evaluation of cardiac function; - non-invasive heart & hepatic iron quantification, by annual Signa-MRI 1.5 Tesla, multi-echo T2 & T2* sequences; - annual endocrinology screening. *Results.* 1) None of the 50 TMp died since combined chelation treatment was implemented, while, with desferrioxamine monotherapy, mortality fluctuated from 13.3 to 14.3% over the last decade. 2) A trend analysis (PROC MIXED in SAS), revealed a negative trend of serum ferritin over time (p<0.0001) with a rate of decline equal to -95 ng/mL/month and a cumulative decrease in 5 years. In the 88.7% of compliant TMps the mean ferritin value at baseline (2.421 μ/L) decreased dramatically (107 μ/L) after 5 years of treatment. 3) In 12 patients with pre-existing heart dysfunction, symptoms (arrhythmias, hypertension and edema) reversed and heart medications were stopped. Ventricular dimensions and function normalized in Echo tests. Mean LVEF increased significantly (p<0.0001) from 54% to 72% following combined therapy. No case of new onset cardiac disease or worsening of pre-existing cardiac dysfunction was evident. 4) MRI measurements (T2Heart & T2Liver) revealed significant reduction of iron overload in both organs over time leading to virtually iron free organs (Table 1).

Table 1.

N=50	Mean T2H	Mean T_2*H	Mean T_2L	Mean T_2*L
Normal values	>35 msec	>28 msec	>33 msec	>25 msec
Desferal® monotherapy	28,2 msec		22,7 msec	
After 3-5ys Combined Chelation	38,1 msec	34,8 msec	37,2 msec	31,7 msec
Difference		9,9 msec		14,5 msec
	p<0,0001		p<0,0001	

5) At baseline, 7 TMps (13%, mean age 38.7 years) had Insulin-dependent Diabetes and 22 (44%, mean age 32.5 years) had Impaired Glucose Tolerance. Following addition of deferiprone, glucose metabolism improved. Insulin production increased and Insulin resistance reduced (Table 2). 6) Reversal of secondary amenorrhea and spontaneous ovulation in individual cases was validated by LH, FSH, E2/Progesterone, ovarian and uterine ultrasound. *Conclusions.* Combined chelation with Desferal® & Ferriprox® seems to be the treatment of choice because of increased efficacy in a minimally intrusive way. The obvious improvement of cardiac function with reversal of cardiac complications and the removal of myocardial iron, led to zero mortality. Not only was abnormal glucose tolerance reversed, but also the cumulative glucose response improved significantly with this regimen. The reversal of secondary hypogonadism and the hope of creating a family improved the quality of life of Thalassaemia patients considerably.

Table 2.

N=22 Abnormal Glucose Tolerance	OGTT: AUC Glucose	OGTT: Mean Fasting Glu	OGTT: Mean 2h Glu	OGTT AUC Insulin	IVGTT Mean Fasting Glu	IVGTT Mean Fasting Ins
Desferal® monotherapy	20322	100.1	174.3	4265	94.5	6.7
After 3-5ys Comb. Chel	16580	81.2	136.6	5231	54.4	8
Difference	3742 p<0.001	18,9 p<0.001	37.5 p<0.001	966 p<0.001	10.1 p<0.003	1.3 p<0.15

0002

PEARSON SYNDROME IN AN INFANT HETEROZYGOUS FOR C282Y ALLELE OF HFE GENE

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Background. Pearson syndrome is a rare mitochondrial disorder characterized by sideroblastic anemia that usually presents since infancy. Liver disease, renal tubulopathy and exocrine pancreas deficiency emerge later in the course of the disease. The syndrome is due to heteroplasmic mitochondrial DNA deletions and rearrangements, the lack of which, however, cannot exclude the disease. Diagnosis is made by clinical criteria and confirmed by genetic findings. *Aim.* To report the second case of Pearson syndrome in an infant heterozygous for C282Y allele of HFE gene. In addition, it is the first reported case successfully treated initially by deferoxamine and subsequently complicated by primary cutaneous zygomycosis. *Case report.* A 2-month old girl suffered from severe anemia since birth. Bone marrow examination revealed ring sideroblasts and signs indicative of dyserythropoiesis. Onset of anemia was accompanied by neutropenia that did not respond to administration of granulocyte colony-stimulating factor. M-FISH (Fluorescent *in situ* hybridization) caryotype was normal. There was neither deletion nor metathesis of any of the chromosomes. Metabolic evaluation was initially normal. Psychomotor development was normal, but the infant grew on the 9th percentile of weight and height. Transaminasemia developed when she was 8 month old, accompanied by thrombocytopenia. Transferrin saturation increased to 84% and ferritin reached the level of 3000 ng/dL. Ultrasonography revealed signs of diffuse-non specific damage of the liver. Deferoxamine was initiated and liver dysfunction subsided. Genetic evaluation revealed that the patient was heterozygous for C282Y allele of HFE gene. Pyridoxine and B12 per os were initiated but the patient did not respond. Corticosteroids were also initiated and the patient initially responded to therapy. One month after the initiation of deferoxamine and corticosteroids, the infant's course was complicated by zygomycosis. Liposomal amphotericin B was initiated while deferoxamine and corticosteroids were discontinued. Ultrasonography revealed that diffuse liver damage had been reversed. Renal tubulopathy presented shortly before discontinuation of antifungal therapy. Enlargement of kidneys and liver was developed. Lactic acid increased (>500 mg/dL) and acidosis became severe. Acute Respiratory Distress Syndrome due to Pneumocystis carinii lung infection evolved rapidly. Neurological disturbances not due to CNS infection developed. Multiple

organ dysfunction followed. The patient died at the age of 13 months. *Conclusion.* Sideroblastic anemia in neonates is unusual and requires specific differential diagnosis. Metabolic disorders are among them and especially those of mitochondria. We report this case as a rare disease having uncommon complications: 1) Pearson syndrome emerged with hematological features until the age of 11 months. 2) Signs of evolution of the disease presented at a time when differentiation from amphotericin-B induced toxicity was difficult. 3) The infant was also heterozygous for C282Y allele of HFE gene. 4) Deferoxamine therapy initially reversed liver damage. 5) High clinical suspicion is necessary for early recognition of rare infections (e.g. zygomycosis). Iron overload, hemochromatosis, deferoxamine and corticosteroids are underlying conditions for developing zygomycosis.

0003

CHELATION THERAPY WITH DEFERASIROX VERSUS DEFEROXAMINE IN TRANSFUSION-DEPENDENT SICKLE-CELL DISEASE: A COST-EFFECTIVENESS ANALYSIS FROM THE US PERSPECTIVE

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Background. Patients with sickle-cell disease (SCD) receiving chronic transfusions require chelation therapy to prevent complications of iron overload. Although deferoxamine is an effective iron chelator, it must be administered as an 8-12 hour infusion 5-7 times per week, leading to poor compliance and/or quality of life. Deferasirox is an once-daily oral iron chelator that produces reductions in liver iron concentrations and serum ferritin similar to those obtained with deferoxamine. *Aims.* To evaluate from a US perspective the cost-effectiveness of deferasirox versus deferoxamine in SCD patients receiving frequent transfusions. *Methods.* Data from a variety of published and unpublished sources were used to estimate the cost-effectiveness of chelation therapy with deferasirox versus deferoxamine in SCD patients receiving frequent transfusions (8 per year). As there are no long-term studies describing the complications of iron overload in patients with SCD, we focused on the short-term (i.e., one year) costs and quality-of-life effects of chelation therapy. We assumed that patients would receive dosages of deferasirox and deferoxamine that have been found to be similarly effective in patients with SCD (17.3 and 36.0 mg/kg/d respectively). To be conservative we assumed that all patients would be fully compliant with chelation therapy and that use of deferasirox therefore would have no effect on risk of complication of iron overload. Cost-effectiveness was measured in terms of the ratio of the difference (deferasirox vs deferoxamine) in costs to the difference in quality adjusted life years (QALYs) over one year of treatment. Unit costs of deferoxamine and deferasirox were based on US wholesale acquisition costs. The cost of deferoxamine administration was based on analyses of health insurance claims data for US patients with transfusion-dependent anemias. Utilities (weights representing patient quality of life) were based on results of a study that used time-trade-off methods to estimate community-based preferences for oral versus infusional iron chelation therapy. *Results.* One year of treatment with deferasirox is estimated to result in a gain of 0.25 QALYs (0.82 vs 0.57 with deferoxamine). If the price of branded deferoxamine is employed, total annual costs were estimated to be $523 lower with deferasirox vs deferoxamine ($29,304 vs $29,827). Deferasirox therefore dominates deferoxamine (i.e., is less costly and results in more QALYs). If the price of generic deferoxamine is employed, costs are increased by $3,527 with deferasirox vs deferoxamine; the cost per QALY gained with deferasirox vs deferoxamine is $13,028. Cost-effectiveness of deferasirox vs deferoxamine was sensitive to the assumed dosages of deferasirox and deferoxamine and the costs and quality of life decrements associated with infusional therapy. *Conclusion.* In patients with SCD receiving frequent transfusions, deferasirox is less costly and yields more QALYs than branded deferoxamine. Compared with generic deferoxamine, the cost per QALY gained with deferasirox versus deferoxamine is well within the range that is generally considered acceptable in the US. Further research is needed to assess the potential implication of deferasirox on the risk-benefit profile of transfusion therapy in patients with SCD.

0004

MANNOSE BINDING LECTIN LEVELS IN THALASSEMIC PATIENTS WITH HEPATITIS C TREATED WITH PEGINTERFERON ALPHA-2?

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Mannose-binding lectin (MBL) is a serum protein belonging to the family of collectin, which plays a critical role in the innate immune response. MBL is an acute-phase reactant of hepatic origin that can bind through multiple lectin domains to repeating mannose and N-acetylglucosamine sugar motifs that are characteristically displayed at high densities on bacterial and fungal cells and on viruses and protozoa but not on mammalian cells. After binding to a pathogen, MBL initiates at least 2 protective functions that are well defined. First, through the lectin pathway, MBL can mediate the activation of the complement system without the participation of antibodies; second, MBL can promote opsonophagocytosis by collectin receptors directly. Experiments *in vitro* and *in vivo* have shown that an MBL deficiency is likely to have a major effect on innate immune activation and appears to predispose individuals to serious infection. The amounts of MBL in human plasma are genetically determined. We studied the effect of treatment with pegylated interferon-a (peginterferon alpha-2α) in MBL levels in regular transfused thalassemic patients with hepatitis C. Fourteen thalassemic patients with hepatitis C were included in the study. Eight with the hepatitis C genotypes 1 and 4 were treated for 48 wks, while 6 with the genotypes 2 and 3 were treated for 36 wks respectively, with subcutaneous infusions of peginterferon alpha-2α (Pegasys, Roche, Basel, Switzerland). MBL levels were measured by means of a fully mechanized latex-particle-enhanced immunonephelometric assay on the BN-100 nephelometer (Dade Behring, Liederbach, Germany). The measurements were performed before and at the end of the treatment with peginterferon alpha-2α. MBL levels were increased significantly in 11/14 patients, independently from the therapeutic scheme, from 2.00±0.21 mg/L to 2.79±0.37 mg/L ($p<0.008$). In the three other patients the MBL levels remained unchanged and relatively low indicating a possible genetic influence. These findings suggest that administration of peginterferon alpha-2α in thalassemic patients with hepatitis C, additional to the reduction of the observed viral load, normalizes the secretion of MBL and thus restore the impaired innate immunity system.

0005

EFFECTIVENESS AND SAFETY OF LONG-TERM COMBINATION IRON CHELATION THERAPY WITH DESFERRIOXAMINE & DEFERIPRONE IN MULTITRANSFUSED PATIENTS WITH THALASSAEMIA

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Background. Transfusional haemochromatosis and increased dietary iron absorption cause severe complications in thalassaemic patients and lead to death before the 3rd decade of life unless treated effectively with iron chelation therapy. In this context, accurate assessment of body iron in these patients is essential for monitoring chelation in order to avoid toxic effects of iron overload and to prevent side effects from hyper doses of the chelator. *Aim.* The aim of this study is to evaluate the efficacy and safety of a combination therapy with the two chelators DFO and DFP, shown to have an additive or synergistic effect when used appropriately in severely loaded thalassaemic patients presenting with cardiac or liver complications. *Methods.* Twelve patients (5 men and 7 women; mean age 36, range 22 to 46 years) have been treated with DFO 40 mg/Kg/day 2 days/week and DFP 75 mg/Kg/day (both chelators used on the same day). Four patients had a medical history of diabetes mellitus type II. Another four had been infected with HCV and two progressed to chronic active hepatitis. Iron load was estimated with serum ferritin and 24-hour urinary iron excretion (UIE) every 3 months. Cardiac and liver function and iron load were measured annually with biochemical tests (ALT, AST and γ-GT), ECHO cardiography and magnetic resonance imaging (MRI-T2). *Results.* Compliance with treatment was very high throughout the study period. No side effects or adverse reactions associated with combination therapy were observed. In patients presenting with cardiac dysfunction before treatment, symptoms disappeared two years after the onset of therapy. As shown in Table 1 serum ferritin decreased significantly ($p=0.010$), while UIE was significantly increased. As regards myocardium and liver, T2 relaxation time was significantly increased ($p=0.002$ and $p=0.048$ respectively), while no significant changes in liver enzymes were observed after treatment. Left Ventricular Ejection Fraction (LVEF) ($p=0.007$) and fractional shortening were significantly increased.

Table 1.

Mean values	*Years*					
	2000	*2001*	*2002*	*20003*	*20004*	*2005*
Ferritin ng/L	7638	4500	2538	2088	1330	1341
ALT i.u/RNA+	210	287	172	174	167	236
ALT i.u/RNA-	100	99	115	119	78	33
MRI T_2 ms Heart	-	-	31.5	38.7	39.9	402
MRI T_2 ms Liver	-	-	20.0	20.1	22.4	23.7
LVEF%	48.9	52.1	55.9	58.7	63.6	64.0
Fractional Shortening%	29.7	30.2	32.5	33.7	35.1	38.7

Conclusions. Our results show high acceptance of long-term combination therapy with DFO and DFP by patients who previously failed to comply with DFO monotherapy. Long-term administration of both chelators used on the same day has been shown to be safe and no deleterious effects were observed. Serum ferritin was positively correlated with cardiac ECHO and MRI. No noteworthy change was found in liver iron, possibly due to the late onset of chelation and consequent permanent liver damage.

0006

CHELATION EFFICACY OF DEFERIPRONE AND DEFERASIROX IN PATIENTS WITH THALASSEMIA MAJOR

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Background. No clinical studies have directly compared the efficacy of the new oral iron chelator deferasirox (Exjade™, Novartis) to that of the first oral iron chelator deferiprone (Ferriprox®, Apotex) in controlling the body iron load in patients with thalassemia major. *Aim.* The relative efficacy of deferasirox and deferiprone can be estimated based on the data currently available for both drugs. Deferasirox data were made available to a US FDA Advisory Committee for a meeting on 29Sep05. We have compared the data of liver iron concentration (LIC) in mg Fe/g dry weight in thalassemia major patients from the Novartis-sponsored pivotal study 0107 for deferasirox and from all patients in the Apotex-sponsored studies for deferiprone who had LIC measurement over the same time interval. *Methods.* The comparison was based on the primary and secondary efficacy criteria utilized by Novartis for the assessment of deferasirox. Primary treatment success criteria were based on the response to therapy in relationship to the pre-existing LIC and defined as: LIC maintained within 1 to <7 for baseline LIC 2 to <7; LIC decreased to within 1 to <7 for baseline LIC ≥7 to <10; LIC decreased by ≥3 for baseline LIC ≥10. The actual change in LIC from baseline to one year of treatment was the secondary endpoint. The dose of deferasirox at study entry was assigned based on the baseline LIC. Patients with baseline LIC of 2-3 received 5 mg/kg/day; LIC of >3-7 received 10 mg/kg/day; LIC of >7-14 received 20 mg/kg/day; LIC of >14 received 30 mg/kg/day. In the Apotex studies, the dose of deferiprone ranged from 75 to 100 mg/kg/day regardless of baseline LIC. Since the FDA concluded that deferasirox was ineffective at the 5 and 10 mg/kg/day doses, this study also compared the efficacy of the two chelators in the sub-group of patients with baseline LIC ≥7 who received the highest doses (20 and 30 mg/kg/day) in the deferasirox group. *Results.* Table 1 provides results on the primary efficacy endpoint for all patients and for those who received the highest doses of deferasirox (LIC ≥7).

Table 1. Success rate based on Exjade™ primary efficacy success criteria.

LIC at baseline	*Success rate (%) after 1 year of treatment*		
	Exjade™ (N=276)	*Ferriprox™ (N=60)*	*p-value*
2-<7 mg Fe/g dw	34/85 (40%)	22/27 (81%)	0.0002
≥7 mg	112/191 (59%)	20/33 (61%)	0.83
Overall	146/276 (53%)	42/60 (70%)	0.016

The results of the secondary endpoint revealed both therapies were associated with a significant reduction in LIC (-2.4±8.2 and -1.4±4.0 for deferasirox and deferiprone respectively; $p<0.001$ for both). The overall reduction was due mainly to the effect of the chelators on the LIC of patients with greater baseline LIC. *Conclusions.* The overall success for the primary efficacy endpoint was greater for deferiprone than deferasirox. The success of the highest doses of deferasirox was similar to that of deferiprone at 75 100 mg/kg/day. Although these data were generated from distinct cohorts of patients participating in independent studies, they represent carefully conducted studies in the same types of patients and provide a means for obtaining an initial comparison. These results highlight the need for a randomized study comparing the two chelators, and one where only effective doses of deferasirox will be used.

0007

THE ROLE OF RETICULOCYTE HEMOGLOBIN CONTENT AS IRON STATUS MARKER IN HAEMODIALYSIS PATIENTS

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Background. Iron deficiency leads the hyporesponsiveness to erythropoietin (rHuEPO) in hemodialysis patients and results in renal anemia. So, the early detection of iron deficiency is of value for the successful treatment of renal anemia. At present, serum ferritin and transferrin saturation (TS) are recommended for assessing iron deficiency. However, they have a limitation in estimating iron status because the lack of accuracy and precision in dialysis patients. The reticulocyte hemoglobin content (CHr) has been proposed as a useful tool in iron status assessment, but its cutoff value for iron deficiency varies from 26 to 32 pg in different studies. *Aims.* We investigate the accuracy of CHr in comparison to the conventional test and a CHr cutoff value. Also, we assess that the CHr change after administration of iron supplement related to changes in red cell count, Hb, and Hct. *Methods.* We selected 163 hemodialysis patients (95 females and 78 males, mean age 56.1±13.2) receiving rHuEPO and oral or intravenous iron therapy. We measured CBC, reticulocyte, CHr (using ADVIA120 autoanalyzer, Bayer Medical, USA), iron parameters (iron, TIBC, ferritin), CRP, BUN and creatinine. Iron deficiency in this study was defined as a serum ferritin < 100 μ/L or a TS < 20%. In patients categorized as iron deficient, CBC, reticulocyte, and CHr were determined at 1 month after iron therapy. *Results.* The mean Hb in hemodialysis patient was 10.0±1.1 g/dL and 53 patients were iron deficient (19 with low ferritin and low TS, 8 with only low ferritin, 26 with only low TS). CHr were distributed with mean 33.9±1.4 pg in iron sufficient group and mean CHr 29.2±1.2 pg in iron deficient group, and showed significant difference between 2 groups. CHr was positively correlated with TS (r=0.36, p=0.02), but there was no correlation with iron, ferritin, BUN and creatinine. The CHr changes were related to changes in red cell count (r=0.13, p=0.045) and Hct (r=0.21, p=0.03). *Conclusions.* CHr is available in measuring iron status in dialysis patients, especially in patients in iron deficiency with normal ferritin. It is considered that the CHr cut-off value 32 pg is appropriate for the assessment of iron deficiency (sensitivity 100%, specificity 80%). Also, CHr might be useful to predict the degree of erythropoietic response after iron administration.

0008

SENSITIVITY ANALYSIS ON THE COST-EFFECTIVENESS OF CHELATION THERAPY WITH DEFERASIROX OR DEFEROXAMINE IN TRANSFUSION-DEPENDENT THALASSEMIA PATIENTS BASED ON EUROPEAN COSTS

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Background. Deferoxamine is an effective iron chelator but must be administered as an 8-12 hour infusion 5-7 times per week, leading to poor compliance, effectiveness, and/or quality of life. Deferasirox is a novel once-daily oral chelator that produces reductions in liver iron concentrations (LIC) and serum ferritin similar to deferoxamine, and has been found to have a favourable cost effectiveness in US thalassemics. Cost-effectiveness in other settings has not been examined. *Aims.* To examine the sensitivity of the cost-effectiveness of deferasirox and deferoxamine among thalassemia patients to costs prevailing in various European countries. *Methods.* A Markov model developed previously for the

US was adapted to examine the potential cost-effectiveness of deferasirox and deferoxamine in various European countries, using ranges of values for costs of chelation which may prevail across these settings. Other inputs were unchanged as they are likely to be similar across settings. Patients were assumed to have thalassemia major, be three years of age at initiation of chelation therapy, and to receive prescribed dosages of deferasirox and deferoxamine that have been shown to be similarly effective in patients with LIC≥7 Fe/g dry weight (24.6 and 47.2 mg/kg/d respectively). Compliance with deferoxamine was based on analyses of health insurance claims data. Because data on compliance with deferasirox versus deferoxamine are unavailable, published data on compliance with the oral chelator deferiprone versus deferoxamine were used. Probabilities of complications of iron overload and death by compliance with chelation were estimated from published studies. Differences in quality of life with deferasirox versus deferoxamine were based on a study of patient preferences for oral versus infusional chelation therapy. The price of deferoxamine was varied from € 15 to € 40 per 2 g vial; the price of deferasirox, from € 40 to € 50 per 1 g vial; and the cost of deferoxamine administration, from € 10 to € 40 per infusion. Costs of complications of iron overload conservatively were not considered. Cost-effectiveness was defined as the incremental cost per quality-adjusted life years (QALY) gained. Future costs and QALYs were discounted at 3% annually.

Results. Compared with no chelation (which yields 7.6 QALYs), deferoxamine yields an additional 4.1 QALYs per patient while deferasirox yields an additional 8.1 QALYs per patient. Expected lifetime costs of chelation therapy with deferoxamine range from € 70,000 to € 226,000 per patient; those for deferasirox range from € 186,000 to € 266,000 per patient. Cost-effectiveness versus no chelation ranges from € 20,000 to € 63,000 per QALY gained for deferoxamine and from € 28,000 to € 35,000 per QALY gained for deferasirox. In almost all scenarios where the cost of deferoxamine administration is € 15 per infusion or more, the cost-effectiveness of deferasirox versus no chelation is more favorable than that of deferoxamine versus no chelation. The cost-effectiveness of deferasirox versus deferoxamine was less that € 50,000 per QALY gained in all scenarios. *Conclusion.* Although analyses based on actual prices of deferasirox are necessary, this analysis suggests that the cost-effectiveness of deferasirox versus deferoxamine or no chelation in European settings is within the range considered acceptable in these countries.

0009

BURDEN OF IRON CHELATION THERAPY SIGNIFICANTLY IMPACTS ADHERENCE TO TREATMENT IN PATIENTS WITH IRON OVERLOAD

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Background. As part of a supportive care programme, thalassemia, sickle cell disease (SCD), and myelodysplastic syndrome (MDS) patients require regular blood transfusions. One consequence of this is iron overload (IO) and, if left untreated, morbidity and earlier mortality. Current iron chelation therapy (ICT) using deferoxamine (DFO) requires 8-12 hour infusions, 5-7 days per week. This potentially limits health related quality of life and inhibits adherence in patients with thalassemia, SCD, and MDS. *Aims.* To assess adherence to ICT amongst patients with thalassemia, SCD, and MDS, and to investigate the extent to which patient adherence with ICT is attributable to socio-demographic and clinical factors, satisfaction, and self esteem factors. *Methods.* Patients with thalassemia, SCD, or MDS currently undergoing ICT completed a 28-item satisfaction with ICT questionnaire comprised of four domains, namely: Perceived Effectiveness, Burden, Acceptability, and Side Effects, as well as three items on adherence to ICT. We analysed data from 110 patients, and performed simple linear regression analyses followed by multivariate regression analysis (with backward selection process) to assess the joint effects of age, whether patients experience side effects, satisfaction with ICT (perceived effectiveness; burden on ICT, acceptability of treatment, and side effects), and self esteem (feelings about yourself). *Results.* The mean age was 30.87 years (SD=14.95). Overall, patients who experienced side effects in the previous 30 days were significantly more likely to think about stopping their ICT than those who did not experience side effects ($p \leq 0.02$). Six variables were identified in the univariate analysis as significant predictors of thinking about stopping medication. Satisfaction with burden on ICT independently explained the most variance and was positively associated with never thinking about stopping medication (higher satisfaction scores were related to never thinking about stopping medication), followed by satisfaction with side effects, acceptability of ICT, age, perceived effectiveness, and then feelings about yourself (Table: Simple regression analyses (R2, *p* values)). The multivariate regression model explained 42.3% of the total variance of thinking about stopping medication. Specifically, a significant positive relationship was demonstrated between never thinking about stopping medication and age ($p=0.04$), perceived effectiveness ($p=0.003$), burden on ICT ($p=0.002$), and satisfaction with side effects ($p=0.01$). *Summary/Conclusions.* This study proposes a framework to understand the complex set of factors associated with adherence to ICT. This analysis shows the following determinants of adherence in order of importance: satisfaction with burden of ICT, satisfaction with side effects, satisfaction with acceptability of ICT, age, perceived effectiveness, and feelings about yourself. These aspects should be considered as part of any supportive care programme. More effective, more convenient ICT will lead to greater satisfaction with therapy and reduce the likelihood of reductions in compliance. Further research is necessary to obtain greater certainty about these relationships and the direction of the relationships.

Table 1.

Significant predictors of never thinking about stopping medication	*Variance explained R^2%*	*p value*
Satisfaction with burden	30%	<0.0001
Satisfaction with side effects	26%	<0.0001
Acceptability	15%	<0.0001
Age	11%	<0.0005
Perceived effectiveness	9%	<0.0014
Feelings about yourself	4%	<0.0436

0010

EFFECTS OF SILYMARIN ON TELOMERASE ACTIVITY AND PROLIFERATION OF? PERIPHERAL BLOOD T-LYMPHOCYTES IN -THALASSEMIA MAJOR PATIENTS?

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Background. Iron, an essential growth trace element, is required for proliferation of all living cells, including T lymphocytes. Many, though not all, immune responses require lymphocyte proliferation. But iron overload, due to diserythropoiesis and regular blood transfusion in β thalassemia major patients, a major problem in Mediterranean as well as in Iran, is associated with impaired lymphocyte proliferative responses to mitogens and cell-mediated immunity. Iron mainly in its non'protein bound, low molecular weight form, cause cellular damage by participating in the generation of the hydroxyl radical, thought to be the principal effector of oxidative DNA damage. One possibility is that telomerase activity, essential enzyme for the repair of telomeric DNA, is reduced following damage by oxygen radicals. *Aims.* The aim of the present study was to investigate telomerase activity in lymphocytes from patients with iron overload disease and to observe its regulation of cellular proliferation and also evaluate effect of Silymarin on this enzyme. *Methods.* Peripheral blood mononuclear cells (PBMC) were isolated from 20 patients with β-thalassemia major and 20 healthy donors. Cells were stimulated with PHA and treated with Deferoxamine and Silymarin for 72 h. Telomerase activity was measured by the telomeric repeat amplification protocol'based telomerase polymerase chain reaction enzyme link immunosorbent assay at 0 and 72 h of incubation. In addition, DNA synthesis of the cells was assayed using BrdU (5-bromo-2'-deoxyuridine) incorporation. *Results.* The results showed that telomerase activity of resting peripheral lymphocytes of healthy subjects and patients with βthalassemia major was detectable at low level, and obviously increased after stimulation *in vitro* with phytohaemagglutinin (PHA) and treatment with Silymarin, and downregulated after treatment *in vitro* with Deferoxamine (DFO). The decreased telomerase activity of resting lymphocytes was found in patients with βthalassemia major compared to that in healthy subjects. The DNA proliferation was paralleled by increase in telomerase activity. *Conclusions.* These results leads to important conclusions. First, the ability of T cells to upregulate Telomerase activity upon activation may decrease over time (aging) and following iron overload-mediated oxidative stress. Second, Silymarin upregulates telomerase activity of T-Lymphocytes and Deferoxamine downregulates. Third There is a direct correlation between telomerase

activity and cell proliferation. One possibility is that telomerase is essential for the repair of telomeric DNA following damage by oxygen radicals. Finally, because telomerase contributes to protection from telomere shortening in activated lymphocytes, it may play a critical role in immune responses and also Silymarin as a Superantioxidant may strengthen immune function through scavengering free radicals and upregulation of telomerase activity. Because immune responses occur in waves of proliferation followed by extensive cell death, the limitation in cell division imposed by oxidative stress-mediated reduced telomerase activity and Telomere shortening may contributes immunedeficiency.

0011

HEPCIDIN MUTATION IN A BETA-THALASSEMIA MAJOR PATIENT WITH PERSISTENT SEVERE IRON OVERLOAD DESPITE CHELATION THERAPY

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Background. Hepcidin is a peptide hormone produced in the liver; it is an important negative regulator of iron absorption from the enterocytes and of iron release from macrophages. Hepcidin dysregulation is implicated in the pathogenesis of several iron disorders. Iron overload and inflammation up-regulate hepcidin synthesis decreasing dietary iron absorption, while anaemia and hypoxia suppress hepcidin expression. Thalassaemia Major (TM) is a hereditary haemolytic anaemia requiring long-life blood transfusions treatment. Iron storage in patients undergoing regular transfusion is responsible for the impairment of heart, liver and hence reduced survival. Iron chelation treatment is required to reduce the morbidity and mortality associated with iron overload secondary to chronic transfusion therapy. *Aim.* Despite regular iron chelation some thalassemia patients have persistent high ferritin levels. To get further insights in this issue, several factors have been investigated including infections, inflammatory status and coexistence of HFE and hepcidin mutations. *Patients and Methods.* We report a case of TM patient with severe iron overload despite regular chelation therapy, carrying mutations in hepcidin and HFE genes. The proband is a 23-years-old homozygous b039 woman, regularly transfused since the age of 1 year, receiving 2-3 units of packed red cells and chelated with Deferoxamine 40 mg/Kg/day 6 days/week. *Results.* The echocardiography showed a slight cardiac left ventricular hypertrophy and ejection fraction of 66%. Hepatomegaly and splenomegaly were found at echography. The patient had hypogonadism and hypotyroidism. Serum ferritin was 4000 ng/mL, transferrin saturation over 110% and NTBI 2,83 microM. Serum prohepcidin value was in normal range (167 ng/mL). Because of severe iron overload, hepcidin and other iron related genes including HFE were analysed. Hepcidin gene was sequenced and a heterozygous '72C>T mutation previously described by Biasotto *et al.* (2004) was identified in the promoter region. HFE analysis revealed a homozygous genotype for H63D mutation. No other mutations were detected in TFR2 gene, ferroportin and HH type 2 gene. *Conclusion.* The '72C>T mutation in hepcidin promoter has been previously reported in subjects with increased iron parameters and is described to aggravate the clinical phenotype and the biochemical indices of iron overload. The coexistence of the b-thalassemia trait with hepcidin mutation and H63D homozygosity could contribute to the development of marked iron overload poorly responsive to chelation therapy.

Anemia/Red blood cells I

0012

EARLY MARKERS OF RENAL DYSFUNCTION IN PATIENTS WITH SICKLE CELL/BETA-THALASSEMIA

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Background. Progressive renal failure is one of the main complications in sickle cell/β-thalassemia (HbS/β-thal). Detection of the progressive renal damage using conventional parameters, such as serum creatinine levels (Cr) or clearance of creatinine (Ccr) is often misleading. The early development of glomerular hypertrophy enhances creatinine excretion and gives false normal results of both Cr and Ccr. Therefore, the renal dysfunction becomes evident rather late. For that reason, the identification of markers that indicate early renal dysfunction as well as further progression to end-stage renal disease is highly desirable. Cystatin C (Cys-C) is a cysteine protease inhibitor, which serves as an endogenous parameter of GFR, while β2-microglobulin (β2-M) is a sensitive marker of the glomerular filtration capacity of the kidney. Finally, N-acetyl-β-D-glucosaminidase (NAG), a widely distributed lysosomal enzyme found predominantly within the renal proximal tubules, is also a sensitive indicator of renal injury. *Aim.* The aim of this study was to evaluate whether Cys-C, β2-M and NAG excretion may serve as early indicators of renal dysfunction in a large cohort of HbS/β-thal patients. To our knowledge, such studies are not available in the literature. *Patients and Methods.* We studied 87 compound HbS/β-thal patients (36M/51F; median age 39 years) and 30 healthy controls. All patients were Caucasians, of Greek origin, had stable disease at the time of evaluation, without sickle-cell crises or infections, and had not been transfused for at least three months before. Serum Cys-C and β2-M were determined by particle enhance immunonephelometry using the Dade-Behring BN Prospec nephelometer (Dade Behring, Liederbach, Germany). Urine NAG activity was measured photometrically at 580 nm using a colorimetric assay (Roche Diagnostics, Mannheim, Germany) and expressed as daily output in U/day. *Results.* Cys-C, NAG and serum β2-M levels were higher in patients than controls ($p<0.01$, <0.0001, and <0.0001, respectively). The incidence of patients with high levels of Cys-C, NAG and β2-M was 32.1%, 74.7% and 70.1% respectively, while only 6.8% of patients had increased serum creatinine levels. Cys-C and serum β2-M showed a strong correlation with Ccr (r=-0.43, $p<0.0001$; and r=-0.38, $p<0.001$, respectively), while NAG positively correlated with proteinuria (r=0.546, $p<0.0001$). An inverse strong correlation was also shown between hemoglobin and β2-M (r=-0.3, $p=0.004$), NAG and Cys-C levels (r=0.315, $p=0.002$). Seven patients with proteinuria received therapy with ACE-inhibitors. Changes of poteinuria positively correlated with NAG levels (r=0.691, $p<0.001$). *Conclusions.* These results indicate that Cys-C is an accurate marker of renal dysfunction, and urinary NAG excretion can be considered as a reliable index of the tubular toxicity, and possible predictor of proteinuria and eventual renal impairment in HbS/β-thal patients. Furthermore, NAG measurement may be used for monitoring ACE-inhibitors therapy in HbS/β-thal patients with proteinuria.

0013

A PHASE I, SINGLE AND FRACTIONATED, ASCENDING DOSE STUDY EVALUATING THE SAFETY, PHARMACOKINETICS, PHARMACODYNAMICS, AND IMMUNOGENICITY OF AN ERYTHROPOIETIC MIMETIC ANTIBODY FUSION PROTEIN, CNTO528, IN HEALTHY MALE SUBJECTS

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Objectives. To assess the safety, pharmacokinetics (PK), pharmacodynamics and immunogenicity of single and fractionated IV doses of an erythropoietic mimetic antibody fusion protein, CNTO 528, in healthy

males. *Methods.* In this randomized, single blind, and placebo (PBO)-controlled study, 44 subjects were enrolled in 5 dose cohorts. In Stage 1, 35 subjects received a single IV administration of 0.03, 0.09, 0.3, 0.9 mg/kg CNTO 528 or PBO. In Stage 2, 9 subjects received fractionated IV administrations of CNTO 528 or PBO on Days 1, 3 and 5 (3 infusions of 0.09 mg/kg or PBO). *Results.* Pharmacodynamics: In subjects treated with IV CNTO 528, a dose dependent increase in reticulocyte counts was observed. The maximum effect occurred at day 8 and returned back to baseline between days 22 through 29. Hemoglobin (Hgb) concentration increased in a dose dependent manner with a maximum effect occurring at day 22. Mean Hgb concentration remained 0.4 g/dL above baseline values at the last measurement, approximately 2.5 months after a single dose administration. A dose dependent increase in RBC count was observed with all RBC indices (MCV, MCH, MCHC) within normal range, indicating an increase in normocytic, normochromic RBCs. In all CNTO 528 treated subjects, a dose-dependent increase in soluble transferrin receptor concentration was observed. A dose-dependent increase in endogenous EPO concentration was observed, followed by a dose dependent decrease in endogenous EPO concentration. Pharmacokinetics: In the single dose part of the study, Cmax and AUC increased in an approximately dose proportional manner. The mean terminal half-life ranged between 6 - 7 days in the higher dose cohorts. Safety: Treatment with CNTO 528 was generally well tolerated. There were no serious adverse events (AEs) and few CNTO 528-related AEs. Two subjects in the highest dose cohort met the protocol pre-specified interruption rule of Hgb > 17.8 g/dL and underwent phlebotomy. In these subjects, high Hgb concentrations were not associated with AEs or clinical symptoms. All AEs were determined by the investigator to be mild to moderate in intensity. The most common AE across all groups was headache, occurring in both CNTO 528- and PBO-treated subjects. There was no dose-related trend across groups, and most subjects who experienced headaches were in the lowest 2 dose groups. There was no indication that any patterns of AEs or significant safety laboratory, vital signs, or ECG abnormalities were associated with the administration of CNTO 528. Immunogenicity: None of the 24 subjects who received single IV administration of CNTO 528 were positive for antibodies to CNTO 528. *Conclusions.* Single and fractionated IV administrations of CNTO 528 were well tolerated and resulted in prolonged, dose-dependent erythropoietic responses with notably low inter-subject variability. PK of IV CNTO 528 was linear and approximately dose proportional. This data provides the first proof of concept in humans for erythropoietic responses and an increase of endogenous EPO levels by an erythropoietic mimetic antibody fusion protein.

0014

CORRECTION OF ANEMIA OF THE POST-OPERATIVE PERIOD AFTER ORTHOPEDIC SURGERY BY ORAL VERSUS INTRAVENOUS IRON VERSUS INTRAVENOUS IRON + EPO: A PROSPECTIVE RANDOMIZED TRIAL

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Background. Approximately 20% of patients after orthopedic surgery (hip or knee replacement) present moderate to severe anemia (Hb between 75 and 105 g/L). They are often treated by oral iron for up to three months. *Aims.* We performed a prospective randomized pilot study to investigate the potential of iv iron or iv iron + EPO to treat this kind of anemia as compared to oral iron therapy. *Methods.* Of the 57 patients included in the study, 47 completed the trial and received either 80 mg of oral iron /day (Tardyferon®) for three months (19 patients), or 200 mg iv iron sucrose (Venofer®) at post-operative-days (POD) 1, 3, 5 and 8 (13 patients), or 200 mg iv iron sucrose (Venofer®) on the same PODs plus 150 IU/kg of EPO (epoietin alpha, EPREX®) on PODs 1, 3 and 8 (15 patients). The patients were followed for three months for hematologic values, iron status and inflammatory parameters. *Results.* At baseline the three groups were similar for all these parameters. In particular, Hb values were 141.3, 133.0, and 134.1 g/L respectively. Nadir post-operative mean Hb values were 98 (POD 10), 91 (POD 8), and 94.5 (POD 8) g/L for the three groups respectively. At POD 20, -Hb (compared to nadir) was 12.5 g/L in the oral iron group, 15.5 g/L in the iv iron group, and 25.5 g/L in the iv iron + EPO group (p=0.016 group 3 versus groups 1 & 2). At POD 30, 'Hb was 27.5, 29, and 32.5 g/L respectively, and 43.8, 48, and 42.5 g/L at POD 90. At day +30, 37%, 46%, and 60% of patients had normalized their Hb value (p=0.0266 group 3 versus groups 1 & 2). At POD 8, the absolute reticulocyte count was 31, 43 and 51 G/L respectively. All patients developed an acute inflammatory state with CRP mean value at POD 1, 8, and 10 of 117, 56, and 34 mg/L respectively. Finally, -ferritin levels at POD 90 were -29, +75, and +84 ug/L respectively (p<0.0005 group 1 versus groups 2 & 3). *Conclusions.* This pilot study clearly shows that in moderate to severe post orthopedic surgery anemia, the highest and most rapid increase in Hb was seen in the group of patients treated by iv iron + EPO. This difference is, in our opinion, due to the acute inflammatory state which developed secondary to surgery and lasted for almost 15 days. The study also shows that therapy with iv iron is well tolerated and, unlike oral iron therapy, allows a complete restoration of iron stores. The impact of this accelerated Hb recovery on quality of life, hospital stay duration and incidence of post-operative complications should be studied in a future trial with a larger patient population.

0015

A NON-INVASIVE EVALUATION OF HEMATOCRIT WITH A NEW OPTICAL SENSOR

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Background. The performance of the NBM-100, a new non-invasive Hematocrit (Hct) measurement device, was tested in primary and secondary medical care environments.The device utilizes a finger sensor (Figure 1) with Occlusion Red/Near-InfraRed spectroscopy (O-RNIRS) technology to non-invasively measure hemoglobin and Hct levels. A non-invasive Hct measurement has many potential advantages including the prevention of pain and potential transmission of infectious diseases, and the reduced need for trained technicians. *Aims.* To examine the clinical utility and accuracy of the non-invasive NBM-100 Hct measurement device. *Methods.* In order to investigate the use of the device in both primary and secondary medical environments, clinical trials were conducted in a blood donation center (USA) and a hematology clinic (Israel). The studies were carried out on a group of 304 subjects (155 Female, 149 Male), ages 18-96 (average age 54.9). All study volunteers were tested non-invasively with the NBM-100 device, and invasively, using a venous sample evaluated on a Cell-Dyn (Abbott) blood analyzer. At the blood bank center, the NBM-100 measurements were performed before donation and the venous blood sample was taken after donation. *Results.* The operating staff at all centers found the NBM-100 easy to use and appreciated by subjects. The venous Hct measurements ranged from 15 to 51%. The mean NBM-100 Hct (36%) was the same as the mean venous result. The standard deviation of the difference between the venous reference and noninvasive Hct readings was found to be 3.3% and the bias between the two methods was 0.40%. The mean absolute error (MAE) was 2.7% and the relative absolute error (RAE) was 7.9%. *Conclusions.* This study supports the use of the NBM-100 device as a useful and accurate non-invasive method for measuring a wide range of Hct in both a hospital (e.g. surgery and blood transfusions) and ambulatory setting (e.g. blood banks). Good agreement was found between the non-invasive NBM-100 and the invasive Cell-Dyn Hct measurements. The non-invasive system was found to be environmentally- and user-friendly by operating staff. These findings support the clinical utility of a commercial application of the NBM-100 device.

0016

BODY IRON BALANCE AND IRON EXCRETION:INTAKE RATIO, ACCORDING TO TRANSFUSIONAL REQUIREMENTS, DURING TREATMENT WITH THE ONCE-DAILY ORAL IRON CHELATOR DEFERASIROX (EXJADE, ICL670)

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Background. In chronically transfused patients it is important to understand how much iron is removed by chelation therapy for a given rate of iron intake, to allow tailoring of treatment regimens to achieve the desired iron balance, ie maintenance in a well-controlled patient or reduction in an iron-overloaded patient. Deferasirox (Exjade®, ICL670) is a novel, once-daily oral iron chelator that was recently approved for the treatment of chronic transfusional iron overload in adult and paediatric patients aged ≥2 years. The efficacy and safety of deferasirox have been established in patients with a range of transfusion-dependent anaemias. Importantly, the efficiency of deferasirox is similar across a wide dose range (~28% over 5-30 mg/kg/day), indicating that the higher the dose, the more iron will be removed from the body. *Aims.* The aim of this post-hoc analysis, which pooled data from four pivotal deferasirox clinical trials, was to evaluate deferasirox in relation to change in net body iron (excretion) and the impact of transfusional requirements (intake) and to determine the iron excretion:intake ratio. *Methods.* A total of 1,005 patients (deferasirox n=652, deferoxamine [DFO, Desferal®] n=353) were stratified according to their transfusional requirements while on study, measured in mL/kg/month of packed red blood cells: <7 (low), 7-14 (intermediate) or >14 (high). In practical terms, 7 and 14 mL/kg/month correspond to approximately 2 and 4 adult units of blood, respectively. In each treatment arm, iron balance was calculated (g/year) and the doses necessary to achieve the desired iron balance were assessed. *Results.* Among the pooled population of deferasirox-treated patients, most (n=419, 64.3%) had intermediate transfusional requirements. When evaluating mean net iron balance and iron excretion:intake ratio in completing patients with baseline and end-of-study liver iron concentration (LIC) assessments and recorded positive iron intake (approximately 90% of overall population), a transfusion- and dose-related response pattern was observed with both deferasirox (n=556) and DFO (n=325). Mean net iron balance results for 10, 20 and 30 mg/kg/day doses are presented in Figure 1.

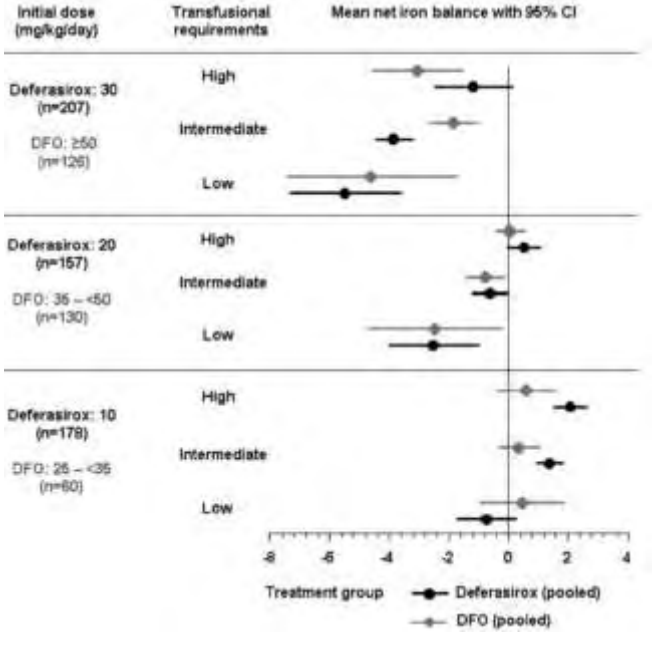

Figure 1. Mean net iron balance (g/year) by treatment, dose and transfusional requirements.

The mean iron excretion:intake ratio was less than 1 (intake exceeded excretion) in all patients receiving deferasirox 5 mg/kg/day, irrespective of transfusional requirements (0.52, 0.67 and 0.34 in the low, intermediate and high cohorts, respectively). *Conclusions.* Based on this analysis, deferasirox 10 mg/kg/day maintained iron balance in patients with low transfusional requirements, 20 mg/kg/day maintained or reduced iron balance in patients with low and intermediate requirements, while 30 mg/kg/day decreased iron balance in most patients, irrespective of transfusional requirements. Since deferasirox efficiency does not vary across doses, it is now known that 5 mg/kg/day is insufficient to maintain or reduce iron balance relative to patients' transfusional requirements. Comparable effects were observed between DFO and deferasirox doses in a 2:1 ratio, indicating that an effective deferasirox dose will be around half that of an effective DFO dose. Deferasirox dosing should therefore be guided by transfusional requirements, severity of iron overload and treatment goal. In addition, as regular transfusions lead to iron accumulation, it is important to monitor transfusion rates, serum ferritin levels and/or LIC.

0017

EPOETIN BETA 30 000 IU ONCE WEEKLY IS EFFECTIVE AND WELL TOLERATED IN ANEMIC PATIENTS WITH SOLID OR NON-MYELOID HEMATOLOGICAL MALIGNANCIES RECEIVING CHEMOTHERAPY: RESULTS FROM THE NAUTICA STUDY

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Background. Anemia is a frequent complication of cancer, affecting about two-thirds of patients at some time during their illness (Ludwig *et al.,* Eur J Cancer 2004). The symptoms of anemia have a profound impact on quality of life (QoL). Low hemoglobin (Hb) levels have also been associated with reduced tumor control and reduced survival. Epoetin β (NeoRecormon®) is an effective treatment for anemia and is equally effective whether administered three times weekly or once weekly (QW) in patients with lymphoproliferative malignancies (Cazzola *et al.,* Br J Haematol. 2003). Few studies, however, have evaluated this QW regimen in patients with a wider range of malignancies. *Aims.* To evaluate the efficacy and safety of epoetin β 30 000 IU QW in patients with solid or non-myeloid hematological malignancies. *Methods.* This was an open-label, single-arm study carried out in 87 centers throughout France between 31 December 2003 and 30 August 2005. Adult patients with solid or non-myeloid hematological malignancies and anemia (Hb levels 8-12 g/dL), a WHO performance status 0-2 and who were scheduled to receive chemotherapy were enrolled. Patients received epoetin β 30 000 IU subcutaneously QW over 16 weeks. Follow-up visits were scheduled after each chemotherapy cycle. The primary efficacy parameter was change in Hb level during epoetin β therapy. Hb response was defined according to patients' baseline Hb level. For patients with Hb levels of 11-12 g/dL at baseline, response was defined as achievement of Hb level of ≥13 g/dL and, for those with Hb levels of <11 g/dL, response was defined as Hb increase of ≥2 g/dL or achievement of Hb level of ≥12 g/dL. *Results.* A total of 691 patients were included in the intention-to-treat population. Mean age was 62.6 (SD, 13.1) years. Fifty-three percent of patients had solid tumors and 47% had hematological malignancies. The mean Hb level at baseline was 10.1 (SD, 1.1) g/dL. The mean Hb level at study endpoint was 12.0 (SD, 2.2) g/dL. The median duration of treatment was 14 weeks. Hb response was observed in 60% of patients during the study and the median time to response was 49 (range 10-130) days. Hb response was seen equally in patients with either hematological malignancies (60%) or solid tumors (61%). Likewise, Hb response was seen with all types of chemotherapy. For the sub-group of patients with Hb <11 g/dL at entry, which corresponds to the intervention level recommended by the EORTC and to epoetin β approved product labeling, the mean Hb increase after 3 weeks was 0.94 g/dL and after 6, 9 and 12 weeks was 1.42 g/dL, 2.03 g/dL and 2.45 g/dL, respectively. Epoetin β treatment was well tolerated. Seven percent of patients presented with thrombo-embolic events, a rate consistent with information provided in the current label for epoetin β. *Conclusion.* In patients with either solid tumors or non-myeloid hematological malignancies, epoetin β 30 000 IU QW effectively and rapidly increased Hb to target levels and was well tolerated.

0018

PATIENT CONTROLLED ANALGESIA VERSUS CONTINUOUS INFUSION OF MORPHINE DURING VASO-OCCLUSIVE CRISIS IN SICKLE CELL DISEASE: A RANDOMIZED CONTROLLED TRIAL

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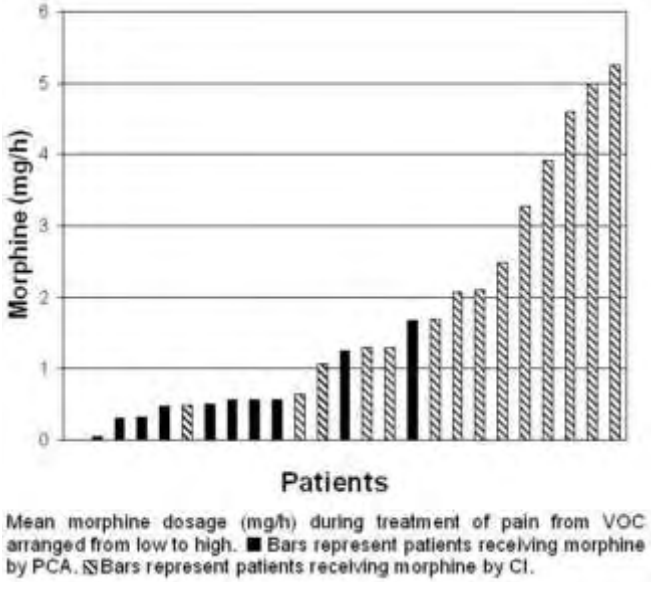
Background. Pain during vaso-occlusive crisis (VOC) in sickle cell disease (SCD) is commonly treated with continuous intravenous infusion (CI) of morphine. During CI the treating physician titrates the dose of morphine until adequate relief of pain has been established. Patient controlled analgesia (PCA) allows the patient to self-administer doses of morphine for the relief of pain and has shown to be equianalgesic in post surgical patients with lower morphine consumption than with the CI of morphine. Morphine has many dose-related side-effects and high plasma levels of morphine are associated with serious complications. *Aim.* To compare the administration of morphine with PCA versus CI in sickle cell patients with VOC we conducted the first randomized controlled trial in this setting. *Methods.* Patients were randomized between PCA and CI of morphine within 24 hours after hospital admission. Endpoints of the study were: the mean and cumulative morphine dose, pain intensity and quality of life (QoL). Pain intensity was measured daily using a ten-point-scale verbal pain score. Reduction of pain intensity was measured by subtracting a pain score on a ten point visual analogue scale (VAS) before randomization from the same measurement two days after randomization. QoL was measured using the Medical Outcomes Study 36-item Short Form Healthy Survey (SF36). *Results.* Twenty-five consecutive episodes of VOC in 19 patients with SCD were included. Patients with PCA demonstrated to have significant lower morphine consumption as compared to patients randomized to CI. The mean and total cumulative morphine dose was 0.5 mg/h and 33 mg in the PCA-group versus 2.1 mg/h and 275 mg in the CI-group, respectively ($p<0.001$ and $p<0.001$). In addition, a non-significant reduction in median duration of hospitalisation was found (6 versus 10). Despite the markedly reduced cumulative dose of morphine in the patients treated with PCA, no difference in pain intensity was found between the groups. The mean daily ten-point-scale verbal pain score was 4.9 in the PCA group versus 5.3, in the CI-group (NS).Also no difference in QoL was found. *Conclusion.* We conclude that the use of PCA in sickle cell patients with VOC results in adequate pain relief at a significant lower morphine dose as compared to morphine administration by continuous infusion.



Mean morphine dosage (mg/h) during treatment of pain from VOC arranged from low to high. ■ Bars represent patients receiving morphine by PCA. ▨ Bars represent patients receiving morphine by CI.

Figure 1. Mean Morphine dosage per patient.

0019

EX VIVO ANALYSIS OF PKLR MUTATIONS THAT AFFECT CORRECT PROCESSING OF PKLR MRNA CAUSING PYRUVATE KINASE DEFICIENCY

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Background. Red blood cell pyruvate kinase (PK) deficiency is the most common cause of nonspherocytic hemolytic anemia due to defective glycolysis. The clinical picture varies from severe hemolysis causing neonatal death to a well compensated hemolytic anemia. PK deficiency is inherited in an autosomal recessive manner and caused by mutations in the PKLR gene. Most of these are missense mutations affecting conserved residues in structurally and functionally important domains of the protein. More rarely, PK deficiency is caused by mutations that lead to aberrant processing of PKLR pre-mRNA. *Aims.* We aimed to study the effect of mutations associated with PK-deficiency and postulated to affect PKLR pre-mRNA processing. *Methods.* Pro-erythroblasts were cultured *ex vivo* from patient-derived $CD34^+$ cells and used as a source of erythroid-specific RNA. We used RT-PCR with fluorescent-dye-labeled primers, fragment analysis, cloning, and DNA sequence analysis of the clones to identify and characterize PKLR transcripts. *Results.* Five different mutations were studied. Two were located at the 5' splice site of intron 3 (c.283G>A) and intron 11 (c.1618_IVS11+1delG). Two mutations were located at the 3' splice site of intron 4 (IVS4-2A>C) and intron 11 (IVS11-3C>G). The fifth mutation was located in exon 8 (c.990C>T). The missense mutation c.283G>A in exon 3 encodes the substitution of glycine by arginine at residue 95. More importantly, this mutation altered the 5' splice site of IVS3. As a result most transcripts did not contain exon 3, coding for a PK monomer that, if translated, lacks amino acids 34 to 94. Similarly, the one-bp deletion at the exon/intron boundary of IVS11, c.1618_IVS11+1delG, altered the 5' splice site of IVS11. This caused skipping of exon 11 in the majority of transcripts, encoding a shortened PK monomer due to a premature end of translation at residue 554. At the 3' splice site, the two main effects of the novel IVS4-2A>C base change were retention of IVS4 and the simultaneous skipping of both exons 5 and 6. Retention of IVS4 predicts the in-frame insertion of 32 additional amino acids between residues 125 and 126. Skipping of both exons 5 and 6 renders a transcript with a premature stop codon in exon 7. The main effect of the IVS11-3C>G mutation was a strongly reduced amount of transcripts. The remaining transcripts were processed normally or at an alternative donor site 5 nt upstream in exon 12. The novel c.990C>T base substitution in exon 8 does not change the codon for serine at residue 330. Interestingly, however, this mutation was associated with an increased amount of transcripts processed at an alternative donor site at nt 985. Consequently, this in-frame deletion would remove residues 329 to 372 from the PK monomer. *Conclusions.* The results of our studies provide insight into the molecular mechanisms by which the herein described mutations lead to PK deficiency. It shows in particular that any type of mutation may affect pre-mRNA processing. This will contribute to the better understanding of the pathophysiology of PK deficiency and, in general, the complex regulation of pre-mRNA processing.

0020

ASSESSMENT OF COGNITIVE EFFECTS OF ONCE-WEEKLY EPOETIN ALFA IN ANEMIC PATIENTS WITH HEMATOLOGIC MALIGNANCIES RECEIVING CHEMOTHERAPY: RESULTS OF THE EPOLYM TRIAL

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Background. Increasing evidence suggests that chemotherapy can produce cognitive dysfunction in cancer patients, and while the cognitive deficits tend to be subtle, they can have a negative impact on the patients' social, educational, and professional activities, and overall quality of life (QOL). Clinical evidence suggests an association between chemotherapy-related decreases in hemoglobin (Hb) level and an increased risk of cognitive dysfunction. *Aims.* To assess changes in cognitive function in patients undergoing chemotherapy and receiving once-weekly (QW) epoetin alfa to maintain Hb levels and prevent subsequent fatigue, symptoms of anemia and deficits in QOL. *Methods.* EPOLYM was a 24 week, prospective, international, multicenter, open-label, Phase IIIb trial in anemic (Hb ≤12.0 g/dL) patients receiving chemotherapy (N=1034) for Hodgkin's disease (HD), non-Hodgkin's lymphoma (NHL), chronic lymphocytic leukemia (CLL), and multiple myeloma (MM). Epo-

etin alfa therapy was initiated at a dose of 40,000 IU QW administered subcutaneously, with dosage adjustments to be made based on clinical response (target Hb, 11.5-13.0 g/dL). Cognitive function was evaluated at baseline, Weeks 1, 6, 12 and at 24 weeks or study completion. Summary statistics were calculated for each measure at each assessment. The Cognitive Drug Research (CDR) Computerised Cognitive Assessment System, an integrated, computerized battery of tests (tasks) performed by subjects, was used to assess changes in parameters of cognitive function, including tasks of attention (Simple Reaction Time, Choice Reaction Time, Digit Vigilance), working memory (Numeric Working Memory), and secondary memory (Immediate and Delayed Word Recognition, Picture Recognition). Because of the relationship between affect and cognition, depression and anxiety were assessed at the time of cognitive assessment using the Hospital Anxiety and Depression Scale (HADS). Changes from baseline in Hb levels, transfusion requirements, and QOL measures were also evaluated (transfusion and QOL results not reported here). *Results.* Analyses were performed on the cognitive data (904/1034 patients) and HADS scores (978/1034 patients). Performance on attention tasks was slightly impaired over the duration of the study, reaching significant decrease from baseline at weeks 12 and 24 ($p<0.05$). Continuity of attention, the ability to sustain attention and avoid error, had a pattern of improvement from baseline over time with a significant improvement at week 12 ($p=0.027$). Speed of memory improved from baseline, achieving significance ($p<0.005$) at each evaluation point. HADS scores were near the high normal range at baseline and improved slightly from baseline during the study, reaching significance ($p<0.001$) from week 6 onward. The baseline up to week 24 significant improvement in HADS scores was associated with increase in Hb level, with those patients achieving an Hb increase of > 1 g/dL with the most improved HADS score. Similarly, the indications of clinical improvement in cognitive function were related to a significant ($p<0.0001$) increase in Hb from 10.4±1.3 g/dL at baseline to 12.0±1.7 g/dL at 24 weeks. *Conclusion.* Overall, the assessment of data indicated a positive change in cognitive function parameters and HADS scores over the 24 week study. These improvements were associated with an increase in Hb level achieved with QW epoetin alfa.

0021

NON-TRANSFERRIN-BOUND IRON AND OXIDATIVE STRESS ARE RELATED TO THE DEGREE OF ERYTHROPOIESIS IN PATIENTS WITH THALASSAEMIA INTERMEDIA

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There is strong evidence that reactive oxygen species (ROS) are involved in the pathogenesis of thalassaemias. It has been shown that ROS are generated in increased amounts in thalassaemic erythrocytes because of the presence and precipitation of excess unmatched globin chains, and deposition of iron, non-haem iron and haemichromes. Continuous ROS production in thalassaemic individuals may alter their overall redox status and cause tissue damage. Reduction in the levels of vitamin C, vitamin E, and carotenoids has been reported in β-thalassaemic patients receiving transfusion therapy. In this study we investigated the oxidative stress in relation to the degree of erythropoiesis in patients with β-thalassaemia Intermedia (TI). Forty patients with (TI) were included in the study. In terms of clinical severity 16 of them were of mild phenotype (non-transfused), while 24 patients were of severe phenotype and only 8 of them were rarely transfused. Non-transferrin-bound iron (NTBI) levels were determined using graphite furnace atomic absorption spectrometry, lipid peroxidation expressed as malonyldialdehyde (MDA) concentration was measured by reverse-phase HPLC with fluorimetric detection and the erythroid marrow activity was estimated by measuring soluble transferrin receptors (sTfR) levels with a turbidimetric technique. The main results of the study showed that a) NTBI and MDA levels were increased (normal controls <0.05 μ/L and <0.65 micromol/L respectively) in 32/40 patients, while sTfR was found 4- to 20-fold higher than normal in all patients, b) NTBI, MDA and sTFR levels were significantly higher in patients with the severe phenotype compared with patients with the mild phenotype ($p<0.01$), c) NTBI correlated positively: with MDA (rho=0.502, $p<0.003$), with sTfR (rho=0.371, $p<0.02$) and Hb F (rho=0.464, $p<0.005$), while no correlation was found between NTBI and Hb levels ($p>0.278$) and d) MDA was correlated significantly with Hb F (rho=0.464, $p<0.005$), while the correlation with sTfR levels was poor (rho=0.313, $p<0.05$). Our findings suggest that ineffective erythropoiesis in patients with thalassaemia intermedia results in increased oxidative stress mediated mainly by increased NTBI levels.

0022

DEFERASIROX (EXJADE, ICL670), THE NOVEL, ONCE-DAILY ORAL IRON CHELATOR, IS WELL TOLERATED AND EFFECTIVE IN TREATING TRANSFUSIONAL IRON OVERLOAD IN PATIENTS WITH A RANGE OF RARE ANAEMIAS

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Background. Deferasirox (Exjade®, ICL670), the novel, once-daily oral iron chelator, is currently approved for use in eight countries for the treatment of transfusional iron overload in patients aged ≥2 years. Deferasirox has been shown to be effective and well tolerated in patients with various transfusion-dependent anaemias, including β-thalassaemia. There are, however, a number of rare anaemias that may also require transfusion therapy, meaning that patients are at risk for iron overload. To date, little has been published regarding iron overload and chelation therapy in these rare anaemias. *Aims.* To evaluate the severity of iron overload, as well as the efficacy and safety/tolerability of deferasirox in transfusion-dependent patients with a range of rare anaemias (a subpopulation of a Phase II study). *Methods.* The overall study was an open-label, multicentre, 1-year trial that enrolled 22 patients with a range of rare anaemias, including: aplastic anaemia (n=5), α-thalassaemia (n=3), sideroblastic anaemia (n=3), myelofibrosis (n=2), pure red cell aplasia (n=2), pyruvate kinase deficiency (n=2), autoimmune haemolytic anaemia (n=1), Fanconi's anaemia (n=1), hereditary sideroblastic anaemia (n=1), erythropenia (n=1), and unspecified anaemia (n=1). Patients were assigned deferasirox doses according to baseline liver iron concentration (LIC). *Results.* Enrolled patients (median age 32 years; range 4-80) received deferasirox 10, 20 or 30 mg/kg/day (n=1, 10 and 11, respectively). The median duration of exposure to deferasirox was 52.1 weeks (range 15.7-66.9). The median number of transfusions during study was 13.5 (25-75% percentiles; 6.0-18.0), while the median blood transfused was 0.31 mL red blood cells/kg/day (25-75% percentiles; 0.12-0.43). Mean baseline LIC in this sub-population was high (15.1 mg Fe/g dw; SD±6.2), but decreased by 3.7 mg Fe/g dw (SD±6.3) after 1 year of deferasirox treatment. Mean serum ferritin level at baseline was 3144 μg/L (SD±1850), and fell by 750 μg/L (SD±1517) during study. The mean rate of iron excretion (0.41±0.19 mg/kg/day) exceeded iron intake (0.31±0.19 mg/kg/day). Seventeen patients (77.3%) completed the study; three subjects discontinued as they no longer required study drug and two withdrew due to adverse events (AEs). There were no deaths in this patient subgroup. All 22 patients reported at least one AE, the majority of which were transient and mild to moderate in severity. The most common drug-related AEs were mild, transient gastrointestinal disturbances such as diarrhoea (n=8, 36.4%), nausea, vomiting (n=4, 18.2% for each) and abdominal pain (n=2, 9.2%). Mild, non-progressive serum creatinine increases >33% of baseline were observed in 12 patients receiving deferasirox 20 and 30 mg/kg/day (within the normal range in seven patients, >ULN in five). There were no incidences of drug-induced neutropenia or arthralgia. *Conclusions.* In these patients with diverse rare anaemias, baseline iron overload was severe and above the published clinically acceptable thresholds. This suggests that these patients are at increased risk for developing co-morbidities with a resultant negative impact on survival. Once-daily, oral deferasirox was effective and generally well tolerated, resulting in a clinically relevant reduction in overall body iron burden.

0023

GLYCOCALYX PERTURBATION IN PATIENTS WITH SICKLE CELL DISEASE: IMPLICATIONS FOR VASCULAR VULNERABILITY

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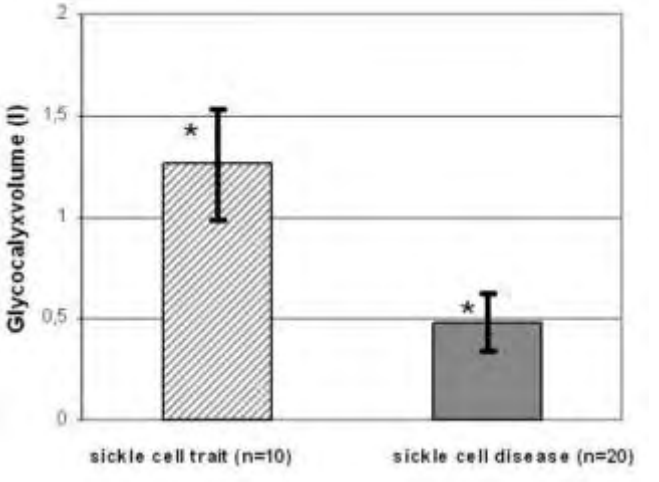
Background. Activated endothelium plays a pivotal role in the pathogenesis of sickle cell disease (SCD). The activation of the endothelium is caused by hypoxia reperfusion damage, high shear rates and pro-inflammatory mediators, like thrombin and TNF-α. The central role of the glycocalyx (a layer of hyaluronan and proteoglycans covering the endothelium) has been established as an antiadhesive and antithrombotic endothelial barrier. Recently, we have validated a technique to determine the total systemic volume of the endothelial glycocalyx in humans and demonstrated that the glycocalyx volume is strongly diminished in diabetic patients with microangiopathy. *Aim.* Sickle cell patients are known to have a strongly activated endothelium and may also develop microangiopathy. Therefore, we assessed the glycocalyx volume in patients with SCD in comparison with carriers of SCD. *Methods.* The gly-

cocalyx was measured in 20 patients with SCD (HbSS, HbSC and HbSβ) and 10 sex- and age-matched carriers of SCD. We determined the total systemic glycocalyx volume by comparing the intravascular distribution volume of a glycocalyx permeable tracer (dextran 40) to that of a glycocalyx impermeable tracer (autologous labelled erythrocytes). *Results.* Patients with SCD demonstrated to have a significant reduced glycocalyx volume of 0.48±0.14 litres as compared to 1.26±0.27 litres in the carriers of sickle cell disease (p=0.009, expressed as mean±SEM). However, no correlation between glycocalyx volume and microangiopathy, disease severity or genotype was found. *Conclusions.* The strongly diminished glycocalyx volume in sickle cell patients resembles the chronic state of activation of the endothelium in SCD that may also may be responsible for the enhanced adhesion of leukocytes and erythrocytes as well as the prothrombotic state of these patients Since the glycocalyx layer serves as an important barrier between the endothelium and the circulating blood cells to prevent the adhesion of leukocytes, therapies that may restore or preserve glycocalyx function are warranted in SCD.



* mean glycocalyxvolume is significantly different between patients with sickle cell disease and persons with sickle cell trait (p=0.009).

Figure 1. Glycocalyxvolumes in litre (mean and SEM).

0024

THE DIFFERENTIAL DIAGNOSIS OF INHERITED SIDEROBLASTIC ANAEMIA

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Background. Over two decades peripheral blood or genomic DNA samples have been referred to University Hospital of Wales for molecular diagnosis of inherited or congenital sideroblastic anaemia (SA). *Aims.* The aim was to review the haematological and additional features of referred cases, to discover the proportion of cases diagnosed, and to critically analyse the features of those cases that remain undiagnosed, in order to identify developmental needs. *Methods.* Routine measurements carried out in our laboratory include FBC, reticulocyte counts, total erythrocyte free (FPP) and zinc protoporphyrin (ZPP), haemoglobinopathy screen, measurement of iron stores and gene sequence analysis of ALAS2, ABC7 and on some occasions FECH. In addition HFE genotyping, erythrocyte mRNA, X chromosome inactivation ratios, globin gene analysis and tissue culture techniques are employed. *Results.* Altogether there are 71 probands (51M, 20F), aged 1yr to >80yr, and 78 relatives of 58 families from 24 different sources around the world. Three were later reported as having other causes including one with Pearson's syndrome. Two were found to have undiagnosed homozygous α thalassaemia (Hb Quong Sze) and were not further investigated. One was not known to have SA and was α3.7kb/-α3.7kb with a lower than expected MCHC. Two others with SA were previously thought to have thalassaemia and one was shown to be α3.7kbαα. In those with raised erythrocyte protoporphyrin levels (22/50) for whom composition was determined (x18) most showed mainly raised free PP (x 13) and only four showed significantly raised zinc PP with absent or only slightly increased free PP. Two had FPP levels in the region expected for erythropoietic protoporphyria (EPP). ALAS2 mutations shown not to be common polymorphisms were found in 25: one new putative GATA-I site 5 nt deletion in the promoter region, two new putative splice site mutations, one single amino acid deletion, and 22 with 16 different missense mutations of which 4 have not yet been described (Ile324Thr; Asp328Glu, Arg368Try, Ser521Phe). Three anaemic female probands carrying novel ALAS2 variations were shown to have skewed X chromosome inactivation in their buffy coat DNA (two have probable severe variations with macrocytic red cells and one with microcytic red cells responded initially to pyridoxine but seems now to be pyridoxine-refractory) and three haematological carriers showed balanced X chromosome inactivation. Variations were found in only four undergoing ABC7 investigations: the published Val411Ile in two brothers (two with raised ZPP) and a maternal uncle with cerebellar ataxia, and a concensus GT→AT splice-site variation of unknown importance in a female heterozygote with severe anaemia. In those patients with markedly raised free erythrocyte PP for whom FECH was examined, only one with values in the EPP region was found to be a double heterozygote for a missense mutation of uncertain importance and the common low-expression allele. *Summary/Conclusions.* These studies extend the range of SA-associated variations, confirm the heterogeneity of presentation and causes and demonstrate a reasonable success for the current diagnostic strategy (approximately 50%). Those who remain undiagnosed remain heterogeneous in presentation requiring new tools to probe the emerging candidate pathways.

0025

INTRAVENOUS ZOLEDRONIC ACID TREATMENT IN THALASSAEMIA-INDUCED OSTEOPOROSIS: RESULTS OF A PHASE II CLINICAL TRIAL

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Background. Osteoporosis is an important cause of morbidity in β-thalassemia patients. Bisphosphonates have been recently used for the treatment of osteoporosis in β-thalassemia. *Aims.* This study is a prospective quasi-experimental study to assess the efficacy and safety of zoledronic acid in thalassemia patients with osteoporosis. The aim of the study is to assess the efficacy and safety of zoledronic acid, administered at a dose of 4 mg intravenously every 3 months over a period of 12 months to patients with β-thalassemia and osteoporosis. *Methods.* Eighteen thalassaemia patients with osteoporosis were given zoledronic acid 4 mg intravenously every 3 months over a period of 12 months. The efficacy of treatment was assessed by measuring Bone Mineral Density (BMD) at the lumbar spine, femoral neck and hip at baseline, 6 and 12 months. Z-score was used to measure the BMD. Other medical assessments included markers of bone formation and resorption (bone alkaline phosphatase (BAP), osteocalcin (OC), and urinary deoxypyridinoline (Dpd)), and the assessment of pain score, analgesic score, and performance score. Ten thalassemic osteoporotic patients were followed up only with serial BMDs as controls.

Table 1. BMD values of treatment and control group.

	Treatment Mean±SD (Z-score)	*p-value*	*Control Mean±SD (Z-score)*	*p-value*
Lumbar Spine				
baseline	-2.84±0.57	<0.01	-2.77±0.78	0.29
6 months	-2.39±0.55		-2.74±0.55	
12 months	-2.20±0.76		-2.92±0.76	
Femoral Neck				
baseline	-1.37±0.74	0.04	-1.75±0.35	0.92
6 months	-1.29±0.66		-1.75±0.21	
12 months	-1.18±0.65		-1.80±0.28	
Total Hip				
baseline	-1.62±0.59	<0.01	-1.60±0.14	0.71
6 months	-1.40±0.68		-1.50±0.57	
12 months	-1.34±0.68		-1.70±0.35	

Results. Both groups had no significant difference with respect to age, gender and baseline BMD. Patients taking zoledronic acid had a significant increase in their lumbar spine, femoral neck, and total hip BMD

measurements over the 12-month period. Patients in the control group did not have any significant change in BMD measurements. Table 1 shows the BMD values of the treatment and control groups.

There was a significant change in the levels of OC and BAP over the 12-month follow-up. There was a significant decrease in the number of painful sites experienced by the patients over the whole treatment period (p=0.01). Pain and analgesic scores significantly decreased over the whole treatment period (p=0.00 and 0.01 respectively). Pain interference with general activity and ECOG score, on the other hand, did not show any significant change. Reported adverse events included joint pain in 9 patients (50%) after the 1st dose and in 2 (11.1%) after the 2nd dose and responding very well to oral analgesics. Two patients (11.1%) had perioral numbness and 3 (16.7%) had low grade fever after the 1st dose. No treatment-related adverse events were reported after the 3rd and 4th doses. None of the patients experienced elevated serum creatinine levels and none discontinued the study. *Conclusions.* Treatment of thalassemic osteoporotic patients with zoledronic acid, administered at a dose of 4 mg intravenously every 3 months over a period of 12 months, is safe and very effective in increasing BMD at the lumbar spine and hip and in reducing pain and is well-tolerated.

0026

THE DISTINCTION BETWEEN HAEMOLYSIS DUE TO HEREDITARY SPHEROCYTOSIS AND THAT DUE TO A CATION PERMEABILITY DISORDER OF THE RED CELL MEMBRANE IN A REGULAR HAEMATOLOGY LABORATORY

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Background. An increased fraction of hyperchromic RBCs, reticulocytosis, splenomegaly and reduced osmotic resistance of RBCs leads in routine haematology to the diagnosis of spherocytosis (HS). If this condition leads to significant symptoms, splenectomy is a possible therapeutic intervention. Splenectomy is not indicated in cases of haemolysis that show cation permeability disorders (CPD), as found in various forms of hereditary stomatocytosis (Stewart *et al.,* Br. J. Haematol 93, 1996, 303-310). We have previously reported a patient, initially diagnosed as having HS, with persistent post-splenectomy haemolysis, in whom further analysis confirmed cryohydrocytosis, a rare disorder with abnormal permeability of the red cell membrane to sodium and potassium (Hematol J., 5, Suppl. 2, 2004, Abstract 250). Based on a detailed study of this patient we designed a simple test to recognize time and temperature-dependent CPD. *Aims.* All blood samples with a significant elevated percentage of hyperchromic RBC were stored on ice for two hours and reanalysed by routine counting in order to detect swelling due to a CPD of the red cell membrane. *Methods.* Routine haematology testing, including reticulocyte counts and erythrocytic histogram with percentage-assessment of hypochromic, hyperchromic, microcytic and macrocytic RBC was performed by flow cytometry (Bayer Advia 120). To assess CPD of the red cell membrane we routinely measured mean cellular volume (MCV), mean cellular haemoglobin concentration (MCHC) and percentage of hyperchromic erythrocytes in whole heparinized blood stored for two hours on ice. *Results.* Approximately one year after the beginning of our study at the University Hospital Zurich, we found a further patient with a mild haemolytic condition who had been splenectomised at another clinic due to the estimated diagnosis of HS. The findings were: Haemoglobin 15.4g/dL (normal range 13.5-17.2), reticulocytes 52‰ (6-17), MCV 95.1fl (80-100), MCHC 39.2 g/dL (31-36), hyperchromic erythrocytes 32.1% (0-1.5), macrocytic erythrocytes 1.8% (0-1.5). Analysis after storage on ice for two hours showed the following values: Haemoglobin 15.6g/dL (normal range 13.5-17.2), MCV 105.5fl (80-100), MCHC 34.7g/dL (31-36), hyperchromic erythrocytes 0.1% (0-1.5), macrocytic erythrocytes 10.9% (0-1.5). Further studies on the patient's RBC revealed that after 2h incubation at 0°C there was a 3.22-fold increase in plasma [K^+]. These data strongly suggested the diagnosis of cryohydrocytosis. The isotopic flux studies at 37°C confirmed an increased oubain+bumetanide-resistant influx of potassium compared to control RBC. The temperature dependence of this leak showed a U-shaped profile with a minimum at about 23°C and a maximum at 0°C. *Conclusion.* Cryohydrocytosis seems to be an underdiagnosed disorder mimicking in daily practice typical hereditary spherocytosis with mild haemolysis. Hence, in cases with suspected HS the regular blood tests should be repeated routinely after a cold storage of whole blood, to exclude or eventually verify cryohydrocytosis.

0027

RITUXIMAB AND FLUDARABINE COMBINATION THERAPY FOR CHRONIC COLD AGGLUTININ DISEASE

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Background. Primary chronic cold agglutinin disease (CAD) is an autoimmune haemolytic anaemia characterized by the production of monoclonal antibodies, most often IgM kappa, against erythrocyte surface antigens. A clonal lymphoproliferative bone marrow disorder can be demonstrated in most cases. Rituximab single agent therapy has been shown in prospective studies to induce remission in more than 50% of patients. *Aim.* We wanted to improve on response rates achieved by therapy directed against the underlying clonal B cell proliferation. *Methods.* In a prospective phase II trial, eligible CAD patients received rituximab 375 mg/sqm intravenously d 1, 29, 57 and 85, and fludarabine tablets 40 mg/sqm d 1-5, 29-33, 57-61 and 85-89. Clinical, haematological, immunological and histological data were recorded, and responses were classified according to previously published criteria as complete (CR), partial (PR), or no (NR) response. *Results.* By Feb 2006 we have treated six patients with a median age of 70. All patients had monoclonal IgM kappa and considerable or severe cold-induced circulatory symptoms. All had been previously treated with rituximab single agent therapy, resulting in one CR, one PR and four NR. After the combination therapy, circulatory symptoms resolved completely in four patients and improved in one additional patient. Haemoglobin levels increased by > 3 g/dL in two of four anaemic patients. Overall, two patients achieved CR, two achieved PR, while two were non-responders. Haematological toxicity was recorded in three patients (grade 2, 3 and 4, respectively), infection grade 2 in one and nausea in one. *Conclusions.* Rituximab and fludarabine combination therapy is feasible even in elderly patients with CAD. Response rates are promising, but superiority over rituximab single agent therapy remains to be proven until more patients have been treated.

Table 1.

Patient	Age	Sex	Indication for therapy[1]	Bone marrow hystology[2]	Hb g/dL	Increase in Hb g/dL	Change in circulatory symptoms	Overall response
1	59	F	HA,CS	LPL	9,9	3,8	Resolution	CR
2	77	F	CS, HA	UCL	10,3	0,3	Improvement	NR
3	66	M	CS	LPL	12,2	1,0	Resolution	PR
4	74	M	CS	LPL	16,0	-0,3	Resolution	CR
5	62	F	CS, HA	LPL	10,4	3,1	Resolution	PR
6	85	M	HA,CS	LPL	7,8	1,18	No change	NR

[1] HA, haemolytic anaemia; CS, circulatory symptoms; [2]LPL, lymphoplasmacytic lymphoma; UCL, unclassified clonal lymphocytosis.

0028

IN VIVO OXIDATIVE ERYTHROCYTE MEMBRANE PROTEIN DAMAGE IN HEREDITARY SPHEROCYTOSIS

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Background. Hereditary spherocytosis (HS) is a heterogeneous group of disorders with regard to clinical severity, gene defects and mode of inheritance. Most patients are presented with mild or moderate hemolysis. The abnormal red cell morphology (resulting in shortened cell survival) is due to a deficiency of, or a dysfunction in, spectrin, ankyrin, band 3 or pallidin. Previous *in vitro* studies suggested that the spherocytes are sensitive to the action of oxidative agents. Furthermore, higher Hb autooxidation rate and abnormal oxidant sensitivity of spectrin, have also been reported in HS. *Aims.* To determine the possible oxidation-related protein alterations and the oxidative index of the membrane ghosts and cytoskeletons in clinically diagnosed cases of HS. *Methods.* Twelve patients with clinical and laboratory diagnosis of mild to moderate HS [ank(-)HS N=4, Sp(-)HS N=3, B3(-)HS N=5, splenectomized N=2, concomitant carriers of α- or β-thalassemia N=4] and twelve healthy subjects used as controls were examined. Total ghosts and cytoskeletons were analyzed by SDS-PAGE densitometry and probed for

hemoglobin, human immunoglobulins (IgG's) and various membrane proteins using erythroid specific antibodies. Carbonylated protein content was determined following 2,4-dinitrophenylhydrazine derivatization and SDS-PAGE coupled with western blotting with anti-DNP moiety antibody. *Results.* Protein degradation, formation of high molecular weight aggregates and increased Hb and IgG's binding to the membrane were found by means of SDS-PAGE and immunoblotting analysis in the majority of the HS patients examined. The protein band-8 (22 kDa) was also increased in 8/12 patients, half of which had concomitantly increase in Hb. Probing of the HS ghost membranes for Hb clarified that the membrane-associated globin was in the form of probably oxidized/denatured Hb or hemichromes. Subsequent analysis of the Triton-extracted membrane skeletons revealed pathologically increased amounts of skeleton-associated Hb monomers and higher order aggregates, representing globin oligomers and complexes with membrane protein components, in 30% of the samples. Immunoblotting with dinitrophenol-specific antibody showed increased RBC membrane and cytoskeleton protein carbonyls in the majority of the HS patients. In comparison to control membranes, there was an evident increase in the number and the intensity of the carbonylated protein bands appearing in the immunostained gels, ranging from MW 240 kDa to 15 kDa. in approximately 70% of the HS samples that were examined. *Summary/Conclusions.* The red cells in HS *in vivo* are characterized by oxidative alterations in Hb and various membrane proteins and increased protein carbonylation levels. Similar defects in thalassemia, ex-vivo stored and senescent RBCs are dictated by increased oxidative stress and are positively correlated with perturbations in membrane properties. These data corroborate the evidence for the occurrence of oxidative damage in membrane proteins in HS and add some new insight in the field of HS pathophysiology.

0029

DARBEPOETIN ALFA ADMINISTERED EVERY 3 WEEKS WITH OR WITHOUT PARENTERAL IRON IN ANAEMIC PATIENTS WITH NONMYELOID MALIGNANCIES RECEIVING CHEMOTHERAPY: INTERIM RESULTS FROM A RANDOMISED OPEN-LABEL STUDY

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Background. Patients with cancer receiving chemotherapy often have chemotherapy-induced anaemia (CIA) and reduced quality of life. Darbepoetin alfa is an erythropoiesis-stimulating agent (ESA) that can effectively treat CIA when administered once every 3 weeks (Q3W). In patients with CIA, limited data in the literature suggest that administration of intravenous (IV) iron with ESA therapy may increase clinical response. *Aims.* This randomised, multicentre, open-label, 16-week study evaluated the safety and efficacy of 500 mcg darbepoetin alfa administered Q3W using the SureClick™ injection device in combination with either IV iron or standard practice for iron administration (oral iron or no iron) in patients with CIA (haemoglobin < 11 g/dL). *Methods.* Patients were randomly assigned to receive either IV iron or standard practice for iron administration. The dose of IV iron was 200 mcg administered either Q3W with darbepoetin alfa Q3W or, if required, as 2 doses (200 mcg total) within a 3-week period. Patients who received 1 dose of darbepoetin alfa and who could have completed the 16-week study period by October 19, 2005 are included in this interim analysis. The planned sample size is 400 patients and accrual will be complete by the time of the conference. Randomisation was stratified by tumour type and baseline haemoglobin (<10 or ≥10 g/dL). The incidence of patient-reported adverse events and serious adverse events, in particular embolic/thrombotic events, was summarised. Efficacy endpoints included the crude% (95% CI) of patients achieving the target haemoglobin (≥11 g/dL) from week 5 to the end of treatment period (EOTP) and the crude% (95% CI) of patients receiving red blood cell transfusions from week 5 to EOTP. Haemoglobin values within 28 days of a transfusion were not included in any efficacy analysis. *Results.* Of the 114 pts included in this interim analysis, 65% were women, 99% were Caucasian, the mean (SD) age was 60 years (12), and 26% had lung or gynaecological tumours. Adverse events were reported by 79% of patients in the IV-iron group (n = 58) and 88% of patients in the standard-practice group (n = 56). Serious adverse events (SAEs) were reported by 31% of patients in the IV-iron group and 38% of patients in the standard-practice group. Treatment-related SAEs occurred in 3% of the IV-iron group and 4% of the standard-practice group; 9% of patients in the IV-iron group and 7% of patients in the standard-practice group had embolic/thrombotic events. Haemoglobin and transfusion endpoints stratified by baseline-haemoglobin category are shown in the table. *Summary/Conclusions.* Based on the interim results, the safety profile for patients receiving 500-mcg darbepoetin alfa Q3W with IV iron appears to be comparable to patients receiving 500-mcg darbepoetin alfa Q3W with oral iron or no iron. The percentage of patients who achieved the target haemoglobin (≥ 11 g/dL) appeared higher, and the percentage of patients who required transfusions appeared lower, in the group receiving IV iron. This trend was consistent in patients in both baseline-haemoglobin groups.

Table 1. Study endpoints.

	IV iron		*Standard <practice*	
	BL Hb <10 g/dL n=22	*Bl Hb ≥10 g/dL n=36*	*BL Hb <10 g/dL n=24*	*Bl Hb ≥10 g/dL n=32*
Mean (SD) BL Hb, g/dL	9.3 (0.54)	10.6 (0.39)	9.1 (0.69)	10.5 (0.47)
Crude % (95% CI) pts achieving target Hb (> 11 g/dL) from week 5 to EOTP	81 (58 to 95)	91 (76 to 98)	63 (41 to 81)	80 (61 to 92)
Crude % (95% CI) pts receiving RBC transfusions from week 5 to EOTP°	19 (5 to 42)	12 (3 to 28)	29 (13 to 51)	20 (8 to 39)

BL: baseline; Hb: haemoglobin; EOTP: end of treatment period; pts: patients; RBC: red blood cell. Note: 115 pts were randomised, but 1 pt in the IV-iron group was not treated.
°Based on the number of pts (for IV iron, n=21 for BL Hb < g/dL and n=34 for BL Hb ≥10 g/dL; for standard practice, n=24 for BL Hb<10 g/dL and n=30 for BL Hb>10 g/dL) who were in the study until at least Day 29.

0030

CHARACTERISATION OF INDIVIDUAL NADH-CYTOCHROME B5 REDUCTASE VARIANTS USING A HETEROLOGOUS EXPRESSION SYSTEM

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Background. Recessive congenital methaemoglobinaemia (RCM) arises from deficiency of NADH-cytochrome b5 reductase (cb5r) and manifests as cyanosis from birth. It exhibits two clinical phenotypes, benign type I and more severe type II, where the cyanosis is associated with neurological impairment. The physiological basis for the phenotypic variation between type I and type II RCM is poorly understood. Several mutations, Arg159del and Val252Met, have been found associated with both types suggesting that it is the combination of both alleles and thus the residual activity of cb5r variants that influences the development of type II as opposed to type I RCM. To date more than 40 mutations of cb5r have been described with a cluster in exon 9 of the DIA1 gene. To characterise individual cb5r variants a heterologous expression system has been developed based upon the X-ray crystallography structure of the rat cb5r protein. Expressed proteins can then be purified to homogeneity and investigated for protein stability, catalytic efficiency, FAD cofactor properties and NADH/NAD+ substrate affinities. *Aims.* To characterise five different cb5r variants, Gly75Ser, Arg159Ter, Asp239Gly, Val252Met, Pro275Leu and Gly291Asp, recently described as causing type I RCM in four patients, who all showed markedly reduced red cell cb5r enzyme activity. *Methods.* The different RCM variants were generated using a bacterial expression system for the soluble, diaphorase domain (residues I33-F300) of rat hepatic cb5r. Four mutants and the wild-type domain were purified to homogeneity and characterized using absorption and CD spectroscopies, initial-rate kinetics of NADH:ferricyanide and NADH:cytochrome b5 reductase activities, thermostability measurements and dye-mediated redox titrations of the FAD prosthetic group. *Results.* Four of the expressed variants, Gly75Ser, Asp239Gly, Val252Met, Pro275Leu and Gly291Asp, were found to

exhibit decreased enzyme activity when compared to the rcombinant wild type cb5r protein. The Arg159del variant protein was unstable and could not be purified thus preventing further characterisation. Although four variants, Gly75Ser, Val252Met, Pro275Leu and Gly291Asp, exhibited impaired protein stability the Asp239Gly had near wild type protein stability. A reduction of 40-fold and 437-fold respectively in the affinity of cb5r towards NADH co-factor was found in the Asp239Gly and Pro275Leu variants. Using predictions from the rat model, residue Asp239 is essential for the selection of NADPH over NADH and Pro275Leu is the main residue required for positioning cb5r to allow binding to the NADH substrate. Although Gly75 is present in a highly conserved area of the FAD-binding lobe of cb5r it appears to influence NADH affinity as the Gly75Ser variant exhibited an increase in affinity for NAD+. *Summary.* The heterologous expression system has been a useful tool for providing insights into the impact of type I RCM mutations on the structure and function of cb5r. It may allow the relationship between the clinical phenotype and cb5r activity to be examined and may lead to better understanding of the pathophysiology of the two types of RCM.

0031

DEFERASIROX (EXJADE, ICL670) PROVIDES 24-HOUR PROTECTION FROM LABILE PLASMA IRON (LPI), IN IRON OVERLOADED β-THALASSAEMIA PATIENTS PREVIOUSLY CHELATED WITH MONO- OR COMBINATION THERAPY

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Background. Chelation therapy aims to reduce iron burden, as patients are at increased risk for developing co-morbidities with a resultant negative impact on survival unless excess iron is removed. This can be achieved by reducing *free* or non-transferrin bound iron (NTBI). LPI, one form of NTBI, is redox-active and can be taken up by cells, resulting in expansion of the cellular iron pool and an increased propensity for radical formation with ensuing oxidative stress. Direct capture of LPI has been suggested to avoid accumulation of cellular iron and to prevent its adverse consequences. *Aims.* To evaluate baseline data from the ongoing ESCALATOR trial and to measure LPI change in a patient subgroup. The overall aims of the ESCALATOR study are to investigate the efficacy and safety of the novel, once-daily oral iron chelator deferasirox (Exjade®, ICL670; 20 mg/kg/day) in 232 iron overloaded β-thalassaemia patients previously chelated with mono-or combination therapy. *Methods.* Patient characteristics (159 aged 2-15 years; 73 aged ≥16 years) were analyzed to determine baseline iron burden. Pre-administration and 2-hour post-administration LPI levels were measured in a subgroup of 14 patients, at baseline and following repeat administration (weeks 4 and 16). *Results.* Despite previous chelation, baseline iron burden in the overall population was very high, indicating severe iron overload; mean baseline liver iron concentration (LIC) was 18.0 mg Fe/g dw (SD±9.1) and serum ferritin was 4148 µg/L (SD±3019), with both measures greater in adult than paediatric patients. Baseline LIC and serum ferritin values were well correlated (R=0.63), supporting serum ferritin as a surrogate marker of body iron burden. In the LPI subgroup, all of whom had received combination therapy with deferoxamine and deferiprone, baseline iron burden was also high (LIC 30.0 mg Fe/g dw; range 11.5-48.9).

Table 1. LPI, pre and post deferasirox administration, at baseline and after repeat administration.*

	Baseline (n=13)		*Week 4 (n=13)*		*Week 16 (n=13)*	
LPI, mmol/L	*Pre*	*Post*	*Pre*	*Post*	*Pre*	*Post*
Mean ± SD	0.99±0.82	0.12±0.16	0.45±0.58	0.08±0.20	0.21±0.27	0.00±0.006
Pre vs post		$p<0.0001$		$p<0.0119$		$p<0.1948$
Baselinevs repeat at pre			$p<0.0187$		$p=0.0007$	

Although baseline LPI levels were high, Table 1 demonstrates a significant reduction in post- versus pre-administration levels at baseline and week 4. Pre-administration LPI levels were within normal parameters (0-0.4 µmol/L) by week 4, and were further reduced by week 16; post- versus pre-administration decreases were therefore less pronounced. Predose levels at weeks 4 and 16 can be regarded as LPI levels achieved at trough deferasirox plasma concentrations; they were significantly lower versus baseline, being close to or within the normal range at weeks 4 and 16. This suggests that deferasirox has potential to provide 24-hour protection from LPI. *Conclusions.* Although all patients had received prolonged mono- or combination chelation therapy, high baseline LIC, serum ferritin and LPI levels indicated clinically significant iron burden. Previous studies suggested that LIC >7 mg Fe/g dw is associated with an increased risk of iron overload-related complications. Based on this analysis, once-daily deferasirox 20 mg/kg/day produces sustained reduction in LPI, which is apparent 2 hours post-administration at baseline and pre-administration at 4 and 16 weeks. Decreased LPI from pre-administration levels at weeks 4 and 16 demonstrates that steady-state deferasirox provides 24-hour chelation coverage with a once-daily dose. Deferasirox also provides 24-hour protection from tissue iron loading and resultant organ damage.

0032

RESIDUAL IRON LOAD ESTIMATED BY T2* MAGNETIC RESONANCE IN EX-THALASSEMIC PATIENTS LONG-TIME AFTER BONE-MARROW TRANSPLANTATION

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Background. Allogeneic BMT is able to cure thalassemia major, but iron depletion therapy is required to remove excess iron that has accumulated before BMT. Data on long-term follow up of iron load indexes in these patients are sparse. *Methods.* Hepatic and cardiac iron load were evaluated with MRI using T2* sequences in 8 ex-thalassemic patients (mean age: 19.5±4.25 years, range: 11-24 years). Their mean age at the time of BMT was 8.25±2.82 years (range: 2.5-12 years). Time that had elapsed from BMT to the present study was 11.3±1.8 years (range: 8.5-13.5 years). Seven out of 8 patients underwent iron depletion therapy consisted of phlebotomy (4 patients), deferoxamine (DFO) (1 patient) and DFO followed by phlebotomy (2 patients) for a mean time of 39 months (range: 14-56 months). *Results.* Mean ferritin levels before BMT were 1748±451 g/L. These levels significantly dropped to a mean of 536±260 g/L at the end of ID therapy. At the time of MRI study, mean ferritin have further improved to 271±253 g/L. Hepatic iron load, estimated by MRI, was moderate in 1 patient, mild in 1, minimal in 4 and within normal range in 2 patients. The patient who had the most severe iron overload (liver T2*: 4.14 msec, ferritin 837 g/L) was not compliant with the prescribed iron depletion therapy. Minimal cardiac iron load was observed in only 2 patients (T2*: 25.6 and 26.4 msec, respectively). The LVEF estimated by MRI was normal (range 56.7-73.4%) in all patients except one, who had a marginal value of 54.1%. *Summary/ conclusions .* Our results showed that most of the ex-thalassemic patients had appreciable residual hepatic iron load after iron chelation therapy, which did not correlate to the ferritin levels. Therefore, more prolonged iron depletion therapy after BMT may be required to achieve complete tissue excess iron removal. Methods, which are more sensitive than ferritin, are required for better iron load estimation in these patients.

Drug resistance & drug pharmacology

0033

GENETIC AND LEUKAEMIA-SPECIFIC FACTORS ASSOCIATED WITH P-GLYCOPROTEIN EXPRESSION AND FUNCTION IN AML BLASTS

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Background. P-glycoprotein (pgp), expressed on acute myeloid leukaemia (AML) blasts, is associated with failure to respond to chemotherapy in AML. *Aims.* This study aimed to determine whether expression and function of pgp may be linked to polymorphisms of the encoding gene (MDR1, also known as ABCB1) and whether leukaemia-specific changes in cell biology may override genetic factors in predicting pgp expression. *Methods.* G1199A, G2677T and C3435T polymorphisms in MDR1 (RFLP analysis) as well as pgp protein expression (using MRK-16) and function (modulation of R123 accumulation by PSC 833) were studied in leukaemic blast samples from 631 patients with AML entered into the NCRN AML14 and AML15 clinical trials. *Results.* 41.7% cases had functional pgp; 42.9% had intermediate/high protein expression; 9.5% had function with undetectable/low protein expression; 12.7% had intermediate or high protein expression with no function and 48% had both low/negative protein and function. At position G1199A there were 5.7% heterozygotes and 0.5% A variant homozygotes. At position G2677T there were 45.7% heterozygotes and 21% TT variant homozygotes. At position C3435T there were 46.7% heterozygotes and 30.5% TT variant homozygotes. In a subset of 316 patients, on whom complete data were available, further analysis was performed. The C3435T and G2677T gene polymorphisms affected pgp protein expression, with the lowest protein expression occurring in the variant TT group for both polymorphisms (using Kruskal-Wallis test, $p = 0.005$ and 0.008 respectively), but there was no significant association between polymorphisms and pgp function. The 1199 polymorphism did not affect protein expression or function. Linkage disequilibrium occurs between positions 2677 and 3435. There was a highly significant association between the haplotype and pgp protein expression (p=0.003). The variant (v/v) homozygote haplotype expressed the least pgp protein (p=0.001). MDR1 mRNA was also measured in 81 patients. Message, protein and function were highly correlated (p<0.001 for each comparison). Biological factors were also analysed. The phenotypic and genotypic factors associated with pgp protein expression are shown in the table. In univariate analysis, white blood cell count, cytogenetic risk group, age at diagnosis, secondary AML/MDS and MDR1 haplotype were all strongly associated with pgp protein expression. In multivariate analysis white blood cell count, cytogenetic risk group, age and MDR1 haplotype retained significance. Cell cycle analysis of 39 consecutive fresh trial samples showed that pgp is associated with a low proportion of cycling cells; median percentage of cycling cells in pgp negative/low samples 4.5%, and in pgp intermediate/high samples 1.0% (p=0.01). *Conclusions.* We conclude that there is an extended MDR phenotype of indolent, pgp positive cells in AML, particularly in the elderly which is affected both by genetic factors and acquired leukaemia-specific factors.

Table 1. Factors associated with pgp protein expression.

	Neg/low pgp protein	*High pgp protein*	*p value*
Median WBC (×10⁹/L)	*32*	*12.5*	*<0.001*
Median age	*57*	*63*	*0.001*
Good risk cytogenetics	*68%*	*32%*	
Intermediate risk cytogenetics	*63%*	*37%*	
Poor risk cytogenetics	*36%*	*65%*	*0.008*
De novo AML	*62%*	*39%*	
Secondary AML	*36%*	*64%*	
MDS	*25%*	*75%*	*<0.001*
2677/3435 haplotype ≥1 WT allele	*56%*	*44%*	
2677/3435 haplotype VAR/VAR	*78%*	*22%*	*0.002*

0034

FCGRIIIA 158 V/V GENOTYPE IS ASSOCIATED WITH INFERIOR RESPONSE TO RITUXIMAB AND CHOP IN PATIENTS WITH DIFFUSE LARGE B-CELL LYMPHOMA

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Background. Patients with follicular lymphoma or Waldenstrom's macroglobulinemia and a homozygous valine(V)/valine (V) at position 158 of the FcγRIIIa (CD16) receptor have a superior response to rituximab monotherapy. This could be related to higher affinity binding of monocytes and NK cells with FcγRIIIa 158 V/V genotype to Fc portion of IgG1, suggesting antibody dependent cellular cytotoxicity (ADCC) as an important mechanism of rituximab action in indolent lymphoma. Similarly, a histidine(H)/arginine(R) dimorphism in position 131 of the Fc_RIIa (CD32) may also be related to the treatment response. There is no data whether these dimorphisms affect response to combination of rituximab and chemotherapy in aggressive lymphoma. *Aims.* We examined the correlation of FcγRIIa and FcγRIIIa gene dimorphisms with response in patients with diffuse large B-cell lymphoma (DLBCL) treated with rituximab and CHOP (R-CHOP). *Methods.* FcγRIIa and FcγRIIIa gene dimorphisms were determined in 46 previously untreated patients with DLBCL presenting with Ann Arbor stage II-IV or extensive extranodal involment (IE). The patients were treated with rituximab (375mg/m^2) and standard CHOP regimen. Genotyping of FcγRIIa 131 histidine(H)/arginine (R) and FcγRIIIa 158 valine(V)/phenylalanine(F) was performed by PCR followed by allele-specific restriction enzyme digestion. Response to R-CHOP was analyzed according to standard criteria. *Results.* Complete or unconfirmed complete remission (CR) after R-CHOP was achieved in 74% patients (34/46). Three patients achieved partial remission and nine patients had no response to the treatment. The frequency of FcγRIIIa 158 V/V, V/F and F/F was 22%, 63%, 15%, respectively. The frequency of FcyRIIa 131 H/H, H/R, R/R was 39%, 48% 13%. There was no difference in age, sex or IPI between the groups. Surprisingly, patients with FcγRIIIa 158 V/V genotype had significantly lower CR rate comparing to FcγRIIIa 158 F carriers (40% vs. 83%, p=0,011). There were no significant differences in CR rate between the patients with different FcRIIa 131 genotypes. *Summary/conclusions.* Contrary to the previous reports of response to rituximab in follicular lymphoma, FcγRIIIa 158 V/V genotype in DLBCL was associated with significantly lower response rate to R-CHOP therapy. These results support the hypothesis that antibody dependent cytotoxicity (ADCC) does not mediate rituximab activity in DLBCL. Some other mechanisms, such as chemosensitization or direct apoptosis may be involved in synergistic effect of rituximab and CHOP in DLBCL. Considering the wide inter-individual variability in pharmacokinetics of rituximab, it is possible that FcγRIIIa 158 V/V genotype provides more effective elimination of rituximab overcoming the hypothetical benefit of ADCC in patients with DLBCL treated with R-CHOP.

0035

DEFINED BONE MARROW NICHE COMPONENTS MEDIATE THE *IN VITRO* RESISTANCE OF AML SAMPLES TO THE TYROSINE KINASE INHIBITOR AG1296 AS WELL AS TO CYTOSINE ARABINOSIDE

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Background. Patients with AML tend to respond well to remission induction chemotherapy, but relapse is frequent, suggesting protection of minimal residual disease cells in the bone marrow niche. *Aims.* We sought to determine the effect of defined bone marrow niche components ' fibronectin and cytokines - on the survival and chemoresistance of AML cells. *Methods.* We examined the effects of the cell adhesion substrate fibronectin and/or the cytokines IL3, IL6, SDF-1, angiopoietin 1, stem cell factor (SCF) and several combinations to the *in vitro* adhesion, survival and response to the tyrosine kinase inhibitor AG1296 and to the nucleoside analogue cytosine arabinoside (ara-C) in 48 hr suspension culture of presentation samples from AML patients. 12 of the 16 samples selected for study had internal tandem duplication of the FLT3 gene, since relapse rates are high in these patients and tyrosine kinase inhibition is an attractive therapeutic strategy. *Results. In vitro* adhesion to fibronectin in primary AML samples at 2 hours was enhanced by co-culture with the cytokines IL-3 (69% increase, p= 0.04) and stem cell factor (89% increase, p=0.04). *in vitro* survival at 48 hours in serum-free sus-

pension culture was enhanced 25% by adhesion to fibronectin (p=0.02, n=10) and further enhanced 32% by IL-3 (p=0.03), and 44% by a four cytokine cocktail (IL-3, IL-6, SCF and angiopoietin 1, p=0.02), but not by SCF, IL-6 or angiopoietin 1 individually. *in vitro* resistance to 15 μM AG1296 was enhanced 18% by adhesion to fibronectin (n=10, p=0.007) and further enhanced 43% by IL-3 (n=10, p=0.005), 37% by IL-6 (n=8, p=0.049) and 88% by the four-cytokine cocktail (n=8, p=0.012). Similarly, *in vitro* chemoresistance to 500 ng/mL ara-C was enhanced 25% by adhesion to fibronectin (p=0.008, n=10) and further enhanced 31% by IL-3 (n=10, p=0.05) and 93% by the four cytokine cocktail (n=8, p=0.017). *Conclusion.* Adhesion to fibronectin increases survival and chemoresistance in AML samples and these effects are enhanced by cytokines, particularly IL-3. The tyrosine kinase inhibitor AG1296 and the cytotoxic drug cytosine arabinoside evoked similar patterns of resistance. It may be necessary to target cell-adhesion-mediated mechanisms of drug resistance in order to prevent relapse in AML.

0036

OVERCOMING CHEMORESISTANCE IN HUMAN CHRONIC MYELOID LEUKEMIA K562 CELLS BY SIRNA INHIBITION OF SHINGOSINE KINASE-1

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Background. Sphingosine-1- phosphate (S1P), the product of sphingosine kinase-1 (SK-1), has been implicated as a second messenger that promotes cellular differentiation, proliferation, migration, cytoskeletal reorganization, apoptosis, cellular proliferation and survival. Many external stimuli, particularly growth and survival factors, activate SK-1, leading to an increase in S1P levels and a concomitant decrease in ceramide levels. The antagonistic effects of these metabolites are regulated by enzymes that interconvert ceramide, sphingosine, and S1P. Thus, conversion of ceramide and sphingosine to S1P simultaneously removes pro-apoptotic signals and creates a survival signal. Inhibition of SK1 by RNAi results in the accumulation of ceramide and sphingosine which induce apoptosis. *Aims.* In this study, the imatinib resistant Ph(+) human CML cells were tried to be sensitized to imatinib by targeting the SK1 gene. *Methods.* The Ph$^+$ human K562 cells were exposed to step-wise increasing concentrations of imatinib. Subpopulations of cells that were able to grow in the presence of 0.2 and 1 μM imatinib, were then selected, and referred to as K562/IMA-0.2 and K562/IMA-1, respectively. Plasmid and siRNA transfection of K562 cells were conducted using an Effectine and DharmaFECT(tm) siRNA transfection reagent, respectively. Caspase-3 activity was determined using caspase-3 colorimetric assay. The mitochondrial membrane potential (MMP) was measured using a JC-1 MMP detection kit. The cellular levels of endogenous ceramides were measured using high performance liquid chromatography/mass spectrometry (LC/MS). The expression analysis of SK-1 was determined by RT-PCR and western blotting. *Results.* Measurement of the levels of SK-1 by RT-PCR and Western blotting, demonstrated that the expression of SK-1 is increased about 2- and 4-fold in K562/IMA-0.2 and K562/IMA-1 cells, respectively, when compared to controls. The possible role of SK1 in resistance to imatinib was further examined by the overexpression of SK1, which increased S1P levels, and prevented apoptosis significantly in sensitive K562 cells in response to 500 nM imatinib at 48 hr. On the other hand, in resistant K562/IMA-0.2 and K562/IMA-1 cells, partial inhibition of SK1 expression by siRNA, confirmed by decreased levels of endogenous S1P, increased sensitivity to imatinib-induced apoptosis. *Summary/Conclusions.* Targeting SK1 pathway, in addition to BCR/ABL kinase inhibition, increased the sensitivity of K562/IMA-0.2 and K562/IMA'1 cells to imatinib while the overexpression of SK1 inreased the resistance in sensitive cells. It was shown that one of the mechanisms responsible for imatinib resistance may be increased amounts of S1P in resistant cells as compared to parental sensitive cells.

0037

DEMONSTRATION OF SYNERGISTIC GROWTH-INHIBITORY EFFECTS OF DASATINIB AND CLADRIBINE IN NEOPLASTIC HUMAN MAST CELLS

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In the majority of patients with systemic mastocytosis (SM) including aggressive SM and mast cell leukemia (MCL), neoplastic cells display the D816V-mutated variant of KIT. KIT-D816V exhibits constitutive tyrosine kinase (TK) activity and has been implicated in malignant cell growth. Whereas wild type (wt) KIT and several non 816-codon KIT-mutants, like KIT-G560V, are sensitive to the TK inhibitor imatinib, KIT-D816V confers resistance against this drug. Therefore, several attempts have been made to identify KIT-D816V-targeting drugs. We show that the novel TK inhibitor dasatinib (BMS-354825) counteracts TK activity of wt-KIT, KIT-G560V, and KIT-D816V as detemined by western blotting. Dasatinib was also found to counteract viability of Ba/F3 cells exhibiting wt-KIT or KIT-D816V, as well as growth of HMC-1.1 cells (KIT-D816V-negative) and HMC-1.2 cells (KIT-D816V-positive), whereas imatinib did not counteract growth of KIT D816V-positive cells over the dose range tested (0.1-10 μM). In all cells examined, the effects of dasatinib were dose-dependent, with 100-1,000-fold higher IC50-values found in cells harboring KIT-D816V compared to cells lacking KIT-D816V as assesed by 3H-Tymidine uptake. The growth-inhibitory effects of dasatinib were found to be associated with induction of apoptosis in HMC-1 cells (detemined by conventional and electron microscopy and by TUNEL-assay). Moreover, in this cell line, dasatinib was found to downregulate the expression of CD2 and CD63, two activation-linked cell surface antigens that are typically overexpressed on mast cells in SM as assesed by flow cytometry. One strategy to optimize the treatment of SM and to overcome drug-resistance might be to combine TK inhibitors with other (targeted or conventional) drugs. We therefore investigated potential cooperative drug interactions between dasatinib and cladribine (2CdA), a cytoreductive agent used for the treatment of SM. Dasatinib was found to synergize with 2CdA in counteracting growth of neoplastic human mast cells harboring KIT-D816V. In summary, our data show that dasatinib *as a single agent or in combination with 2CdA* counteracts growth of neoplastic mast cells and may thus represent a promising new candidate drug for the treatment of SM.

0038

SENSITIZING LEUKEMIC CELLS TO GLUCOCORTICOSTEROIDS BY INHIBITING NFKB ACTIVATION

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Glucocorticoids (GC) are commonly used in childhood leukemia, and induce apoptosis in GC-sensitive leukemic cells. Resistance to GC is a major adverse prognostic factor, occurring in ±20% of newly diagnosed childhood acute lymphoblastic leukemia (ALL) and in >50% of relapsed ALL, while acute myeloid leukemia (AML) is largely unresponsive to GC. Nuclear factor -kappaB (NFkB) is a transcription factor regulating the expression of cell survival genes, counteracting GC-induced cytotoxic effects both directly, via binding to the glucocorticoid receptor (GR) and indirectly. Blocking NFkB might prove a successful strategy to increase glucocorticoid sensitivity of leukemic cells. Chronic exposure of Cem-C7 T cell leukemia cells to sulfasalazine (SSZ), an inhibitor of NFkB activation, sensitized these primary sensitive cells even 10-20 fold further for GC (Van der Heijden *et al.,* 2004). Two myeloid leukemia cell lines, THP1 and U937, with inherent resistance to GC (IC50 for dexamethasone >10 μM) were also markedly sensitized for GC upon long-term exposure to SSZ (IC50 <0.1 μM). In SSZ-exposed cells GR, NFkB p65 and IkB protein expression was markedly increased. Expression of NFkB p50 remained unaltered, suggesting an inactive state of NFkB, which was confirmed by an NFkB activity assay. mRNA levels of all tested genes remained unchanged, suggesting that the GC-sensitizing effect is due to diminished post-transcriptional protein degradation. Consistently, co-incubation with a proteasome inhibitor further enhanced (5-fold) GC sensitivity in SSZ-exposed cells. To determine whether GC sensitization could also be achieved in cells that became GC-resistant due to previous GC exposure, we tested the GC sensitive CEM cell-line C7H2 and six CEM-C7H2 sublines with acquired GC resistance after GC exposure. All cells were exposed to SSZ, after which dexamethasone (dex) sensitivity was measured. Three cell-lines (IC50 dex >11.6 μM) gained increased GC sensitivity (IC50 dex 1.7-3.0 μM). One cell-line (IC50 dex >6 μM) remained resistant (IC50>6 μM) although some degree of sensitization could be seen (IC20 from 2.43 μM to 0.46 μM). Two cell-lines only showed a transient increase in GC sensitivity. We are currently measuring the potential GC sensitizing effects of the proteasome inhibitor Bortezomib since it has been shown that this drug inhibits NFkB by inhibiting the degradation of IkB. In addition, we have found earlier that co-incubation with Bortezomib further enhanced (5-fold) GC sensitivity in SSZ-exposed cells. These experiments will be per-

formed both in cell lines and primary patient samples. In conclusion, several GC-resistant cell-lines, both of lymphoid or myeloid origin, could be sensitized to GC by NFkB inhibitor sulfasalazine. SSZ could also diminish GC resistance that was acquired after GC exposure, an event often seen in the clinic. Further investigations are warranted to establish whether therapeutic strategies targeting NFkB could be exploited to (re)sensitize (relapsed) childhood ALL or even AML for GC.

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0039

RAPAMYCIN OVERCOMES DEXAMETHASONE RESISTANCE OF MALIGNANT PLASMA CELLS

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Background. In multiple myeloma, several promising new agents with mechanisms of action different from standard chemotherapy have been developed during the past few years. These agents may be able to improve the outcome of multiple myeloma, especially if they are able to enhance the response towards conventional therapeutics when given in combination. *Aims.* In order to provide rationale for combination therapies, we performed *in vitro* studies on the effect of combinations of conventional and novel drugs on human multiple myeloma cell lines. *Methods.* Two cell lines, JK-6L and L363, were incubated with various concentrations of drugs, alone or in combination, in the presence or absence of human bone marrow stromal cells (BMSC). Cell growth was measured in an MTS assay and results were evaluated for synergism. *Results.* In the absence of BMSC, dexamethasone (Dex) and rapamycin (Rapa) inhibited the growth of JK-6L cells in a synergistic fashion (e.g. 0.4 µM Dex reduced cell growth to 89% of that observed in untreated controls, 0.25 nM Rapa reduced growth to 56% of untreated controls, combining both agents reduced growth to 27%), whereas no synergism was observed for the L363 cell line. Interestingly, in the presence of BMSC, growth of both JK-6L and L363 cells was blocked in a synergistic fashion. Closer examination of the results from the cell growth assays revealed that JK-6L cells are resistant to Dex, regardless of the presence or absence of BMSC. Rapa was able to overcome this resistance (i.e. in the presence of Rapa, a strong dose-dependent reduction of cell growth by Dex was observed), hence explaining the observed synergy between both compounds. In contrast, L363 cells were already Dex sensitive in the absence of BMSC, and sensitivity was not enhanced by the addition of Rapa. However, in the presence of BMSC, L363 cells became Dex resistant and treatment with Rapa was able to overcome this protective effect and, as in JK-6L cells, restored Dex sensitivity. Furthermore, we determined the rate of apoptosis, upon treatment of JK-6L cells with either agent alone or in combination, by Annexin V (AxV) staining. Addition of Rapa (which by itself did not alter the apoptotic rate) was able to enhance the cytotoxic effect of Dex, confirming the data obtained from the cell growth assays. *Summary/Conclusions.* Rapa synergizes with Dex by overcoming the inherent (JK-6L) or BMSC dependent (L363) Dex resistance of malignant plasma cell lines. This *in vitro* study provides rationale to explore the use of combinations of these agents in patients with multiple myeloma.

0040

MULTIDRUG RESISTANCE MECHANISMS IN HUMAN CHRONIC MYELOID LEUKEMIA CELLS

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Background. Chronic myeloid leukemia (CML) is diagnosed by finding a specific translocation between chromosomes 9 and 22. The resulting Bcr-Abl codes for a fusion protein with tyrosine kinase activity leading to uncontrolled cell growth. Imatinib, a Bcr-Abl inhibitor, induces apoptosis in CML cells by stabilizing the non-ATP-binding form of Bcr-Abl, and in turn, phosphorylation of its substrates. *Aims.* Despite the excellent clinical results with imatinib in CML, most patients have minimal residual disease and others will develop resistance which may eventually progress. In this study, the mechanisms responsible for imatinib resistance was investigated. *Methods.* The Ph+ human K562 and Meg-01 cells were exposed to step-wise increasing concentrations of imatinib. Subpopulations of cells that were able to grow in the presence of 0.2 and 1 µM imatinib, were then selected, and referred to as K562/ or Meg-01/IMA-0.2 and Meg-01/IMA'1, respectively. The IC50 values were determined from cell survival plots obtained by MTT. The expression patterns of Bcr-Abl, MDR1 and apoptotic proteins were detected by RT-PCR and western blotting. Caspase-3 activity was determined using the caspase-3 colorimetric assay. Mitochondrial membrane potential (MMP) was measured using a JC-1 MMP detection kit. Cell cycle profiles of cells were analyzed by flow cytometry. *Results.* K562/IMA-0.2, K562/IMA-1, Meg-01/IMA-0.2 and Meg-01/IMA-1 expressed about 2.3, 19-, 2- and 5-fold resistance to imatinib, as compared to their parental counterparts. There were an icreased expression of Bcr-Abl, MDR1, Bcl-2, and Bcl-XL and decreased expression of Bax protein in resistant cells as compared to their parental counterparts. A decrease in caspase-3 activity and an increase in MMP was detected in resistant cells comparing to parental cells. Exposure to 500 nM IMA for 48 hr resulted in apoptosis in about 75% and 60% of the population in K562 and Meg-01 sensitive cells, while there were no apoptosis in K562/IMA-0.2 and only 20% of apoptosis in Meg-01/IMA-0.2 cells. *Summary/Conclusions.* Various diverse mechanisms have been reported for their involvement in the multidrug resistance. In this study, it has been well documented that the degree of BCR/ABL expression appears to be directly proportional to the levels of imatinib resistance. In addition, there have been BCR/ABL-independent mechanisms reported for deriving resistance against imatinib. Our results revealed that besides Bcr-Abl overexpression, imatinib resistance also depends on the inhibition of apoptosis as a result of up-regulation of anti-apoptotic Bcl-2 and Bcl-XL proteins, down-regulation of pro-apoptotic Bax protein, decreased caspase-3 activity, and increased MMP K562/ or Meg-01/IMA-0.2 and Meg-01/IMA-1 cells.

0041

IDENTIFICATION AND CHARACTERIZATION OF A HOMO-DIMER OF ABCG2 IN MATURE HUMAN ERYTHROCYTES

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Background. Human ATP-binding cassette G2 (ABCG2, also known as breast cancer resistance protein, mitoxantrone resistant protein, and ABC placenta) is a member of the ATP-dependent binding cassette (ABC) family of transporters. Similar to other well characterized ABC transporters that are expressed in humans, namely P-glycoprotein (P-gp) and Multi-Drug Resistance Protein 1 (MRP1), ABCG2 has been shown to transport xenobiotics and other normal cell metabolites and anti-cancer drugs. ABCG2 is termed a half-transporter as two 70-kDa halves are necessary to form a fully active transporter, although recent evidence suggests that the protein may behave as multiple homo-dimers together, i.e. as a homo-tetramer. ABCG2 has also been implicated in the transport of heme, and enhances hypoxic cell survival through interaction with heme. ABCG2 has been found to be expressed in hematopoietic stem cells and erythroid cells, however little is known about the expression and activity of ABCG2 in mature erythrocytes. *Aim.* To characterize the ABCG2 homo-dimer in mature erythrocytes. *Methods.* Erythrocyte plasma membranes were isolated from whole blood drawn from consenting blood donors. ABCG2 protein was visualized using western blotting technique. Activity was determined using FACS analysis whereby the active transport of Pheophorbide a (a chlorophyll catabolite, similar in structure to Protoporphyrin IX) was inhibited using an ABCG2 specific inhibitor Fumitremorgin C. *Results and Conclusion.* In this report, we present the first evidence of an ABCG2 homo-dimer expression in erythrocytes. It appears that the levels of ABCG2 oligomerization varies in human erythrocytes, however, no correlation was found between expression levels from genders, different blood types, as well as samples from different racial groups. The protein was found to be active as demonstrated using FACS analysis to transport Pheophorbide a (a chlorophyll catabolite, similar in structure to Protoporphyrin IX) and the subsequent inhibition of transport of Pheophorbide a was achieved using an ABCG2 specific inhibitor Fumitremorgin C. The results show that ABCG2 in erythrocytes have more of the homo-dimer as compared to MCF7/Mitoxantrone resistant cell line. This is likely due to the formation of di-sulfide bonds resulting from a highly oxidative environment in erythrocytes. The biological significance of this finding is not clear at the present time but may contribute to the physiologic function(s) of ABCG2 in erythrocytes and its possible role in maintaining heme homeostasis. Further studies to characterize the possible functions of ABCG2 in mature erythrocytes are currently in progress.

0042

FUNCTIONAL AND GENOME-WIDE ANALYSIS OF ACQUIRED RESISTANCE TO TRAIL/APO2L MEDIATED APOPTOSIS OF HL60 LEUKEMIA CELLS

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Background. Acute leukemia comprises malignant diseases of clonal character, to which specific treatment remains limited. Apoptosis induced by death receptor activation (i.e. by tumor necrosis factor-related apoptosis inducing ligand, TRAIL/APO2L) is a potential anti-tumor therapeutic mechanism. TRAIL, a member of the TNF family of death ligands, appears to specifically and efficiently kill tumor cells of diverse origin while sparing normal tissues. The TRAIL receptor family consists of five receptors: two death receptors (DR4/TRAIL-R1, DR5/TRAIL-R2), two decoy receptors (DcR1/TRAIL-R3, DcR2/TRAIL-R4), and osteoprotegerin (OPG). *Aims.* Functional analysis of individual TRAIL receptors in HL60 myeloid leukemia cells and analysis of the molecular basis of TRAIL resistance. MATERIALS AND *Methods.* TRAIL-resistant cells were selected from the original HL60 population using pressure of recombinant His-tagged TRAIL (200-2000 ng/mL). The expression of TRAIL receptors and CD14 were analyzed by flow cytometry using fluorochrome labeled antibodies and/or by real-time RT-PCR. Percentage of apoptotic cells was measured by flow cytometry using Annexin-V-FITC/Propidium iodide apoptosis detection kit. The contribution of individual TRAIL receptors on the transmission of apoptotic signal was measured using blocking antibodies to TRAIL receptors. The TRAIL resistance related genome aberrations were analyzed by genome-wide loss of heterozygosity (LOH) screening with marker density of 10cM and comparative genomic hybridization (CGH) assay. *Results.* The blockage of DR4 receptor significantly reduced the number of apoptotic HL60 cells compared to untreated controls. The blockage of DR5 receptor also inhibited TRAIL-induced cell death but the results did not reach statistical significance. Combination of anti-DR4 and anti-DR5 antibodies almost completely abrogated TRAIL-induced HL60 cell death and significantly reduced apoptosis compared to control or anti-DR4 antibody alone ($p<0.01$). Blocking of decoy receptors (DcR1, DcR2) or OPG of HL60 TRAIL-sensitive and TRAIL-resistant cell lines did not significantly affect the apoptotic signaling. Two distinct HL60 TRAIL-resistant phenotypes were identified based on the expression of TRAIL-receptors and CD14. *Phenotype-1* (n=4) was characterized by the decreased expression of TRAIL receptors DR4, DR5, DcR1, and DcR2, CD14 and unchanged expression of OPG as compared to control TRAIL-sensitive HL60 cells. *Phenotype-2* (n=3) was characterized by the decreased expression of DR5 receptor, increased expression of CD14, and undetectable expression of OPG compared to control TRAIL-sensitive HL60 cells. Using LOH assay we identified two genotypes. The first exhibiting deletion on the short arm of chromosome 1p22 and monosomy of chromosome 18, and the second had deletions/uniparental disomy on the short arm of chromosomes 2, 3, 6, and 14. The identified genotypes corresponded to TRAIL-resistant *phenotype-1* and *phenotype-2*, respectively. CGH assay confirmed the loss of genomic material of whole chromosome 18. Further, the CGH detected a gain of genomic material at 1q21-23 of TRAIL-resistant *phenotype-1* while the *phenotype-2* cells did not show genomic defects of chromosome 1. *Summary/Conclusions.* In HL60 cells TRAIL-specific apoptotic signal is transduced predominantly through TRAIL receptor DR4. Decoy receptors, including OPG, did not play a role in TRAIL resistance. The identified TRAIL-resistant phenotypes are associated with distinct genomic conditions. Supported by: IGA MZ NR8317-4 and GAUK 50/2004/c.

0043

THE ADMINISTRATION OF REPEATED DOSES OF CYCLOPHOSPHAMIDE, INDUCES CYTOCHROME P450 IN RAT

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Background. Cyclophosphamide is used in high doses as a part of the conditioning regimen prior to stem cell transplantation. It is usually given for two or four consecutive days, primarily to facilitate engraftment of donor cells. Cyclophosphamide is a prodrug that has to be activated in the liver by a 4-hydroxylation reaction catalyzed by cytochrome P450 (CYP) enzymes. Several studies have shown that cyclophosphamide induces its own metabolism, which affects its pharmacokinetics and pharmacodynamics after repeated doses. *Aim.* In the present study, we aimed to investigate the effect of repeated doses of cyclophosphamide on the CYPs in rat. The levels of mRNA, protein, and enzyme activity were investigated. *Methods.* Male Wistar rats were given 4 consecutive doses of CPA (2 dose levels). Plasma and livers were collected to study the pharmacokinetics of cyclophosphamide in plasma and to measure the levels of mRNA (by real time PCR), protein (by western blot) and enzyme activity (by microsomal incubation with cyclophosphamide) of CYPs, respectively. *Results.* mRNAs of CYP2B1 and 2B2 were significantly induced with repeated dosing. Protein levels were also induced and autoinduction of CPA metabolism to 4-hydroxylation was found. *Conclusion.* Repeated dosing of CPA leads to autoinduction of CPA metabolism and induction of CYP2B mRNA and protein in rat. This knowledge may help in optimizing the dosing regime of cyclophosphamide in patients to keep plasma levels within the therapeutic range. It may also help in minimizing drug-drug interactions and hence increase the therapeutic efficacy and reduce side effects of cyclophosphamide in cancer patients.

0044

OPTIMIZATION OF THERAPY FOR THIOPURINE S-METHYLTRANSFERASE DEFICIENT CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA PATIENTS

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Background. Thiopurine S-methyltransferase (TPMT) is an enzyme that inactivates thiopurine drugs, such as 6-mercaptopurine (6-MP) and thioguanine, commonly used in therapy for childhood acute lymphoblastic leukemia (ALL). In BFM protocol for childhood ALL, 6-MP is administered during maintenance therapy. Patients with low TPMT activity experience severe hematological toxicity when standard 6-MP doses are used. It is now well established that lower TPMT activity can be due to TPMT gene mutations. Three alleles account for more than 95% of the clinically relevant TPMT variants: TPMT*2, TPMT*3A and TPMT*3C. Wild type has been designed as TPMT*1. TPMT*2 allele contains single G238C mutation, TPMT*3C-A719G mutation, TPMT*3B-G460A mutation and TPMT*3A allele has two mutations (G460A and A719G). *Aim.*The purpose of this study was to determine the relevance of TPMT gene mutations in the management of childhood acute lymphoblastic leukemia (ALL). *Methods.* Blood samples from 100 children with ALL were analyzed for TPMT mutations, using polymerase chain reaction-based assays (PCR-RFLP and ARMS). For 50 patients TPMT variant alleles were determined retrospectively, after completing the standard BFM protocol maintenance therapy. Maintenance therapy period was compared according to patients' TPMT genotypes. For the other 50 patients TPMT variant alleles where determined prospectively. For prospectively detected patients with TPMT variant alleles we introduced therapy protocol modification in a way that if leucopenia was noticed, only the dose of 6-MP was reduced but there were no reduction of Methotrexate (MTX) doses. The number of weeks when full, reduced dose or no 6-MP therapy was given, was determined for each patient during the maintenance therapy. Number of neutropenic fever was also considered as a toxic effect of 6-MP therapy. *Results.* Of 100 patients participating in this study, 89% were homozygous for TPMT*1 (W/W), 10% were heterozygous (W/M): 9% for TPMT*1/*3A, 1% for TPMT*1/*2. One patient was double heterozygous for TPMT*3A/*3B

(M/M). Among 50 patients retrospectively analyzed for TPMT variants 6 were found to be W/M. Mean duration of full dose therapy was significantly longer (p<0.01) in W/W patients (54 weeks) than in W/M (37,5 weeks). Mean duration of period with no therapy was significantly longer (p<0,01) in W/M patients (11,3 weeks) than in W/W (3,4 weeks). Neutropenic fever occurred in all of the patients (1-4 times). For four prospectively detected W/M patients, therapy protocol was modified (dosage reduction of 6-MP by 25-50%). In contrast to W/M patients retrospectively analyzed, these W/M patients neither missed the therapy nor developed febrile neutropenia. *Conclusion.* The ability to tolerate 6-MP based maintenance therapy was used as a surrogate marker of hematological toxicity in childhood ALL. We found that even patients heterozygous for TPMT variant alleles are at greater risk of thiopurine drug-related leucopenia. Lowering doses of 6-MP in heterozygous TPMT deficient patients while allowing administration of full dose of MTX, might be an optimal way of treatment for this group of patients. These results justify performing TPMT genotyping before initiating thiopurine therapy in all children diagnosed with acute leukemia to minimize consequent toxicity.

0045

IMATINIB AND HUMAN ORGANIC CATION TRANSPORTER 1 (HOCT1): CHARACTERISATION OF TRANSPORT IN STABLY TRANSFECTED MYELOID CELLS

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Imatinib is an important drug for treating chronic myeloid leukaemia (CML). However, not all patients achieve a major cytogenetic response (MCR), while others lose a MCR. We have been interested in the cellular uptake and efflux of imatinib, and particularly whether this may influence clinical outcome. A previous study in our lab showed that the influx of imatinib is mediated by hOCT1 while efflux is through MDR1 (Thomas *et al.,* Blood, 2004; 104: 3739-3745). We also analysed the expression levels of hOCT1 and MDR1 in 67 imatinib-treated CML patients. Forty patients achieved a MCR (of which 33 were complete) and 27 had no cytogenetic response (NCR) after 6 months of imatinib. Prior to commencement of imatinib, hOCT1 expression levels were greater in patients destined to achieve MCR than in NCR patients. MDR1 expression was high in initial MCR patients who subsequently lost their cytogenetic response, and most of these had developed a BCR-ABL kinase domain mutation at progression. These data are compatible with the view that hOCT1 expression prior to imatinib is an important determinant of the outcome of imatinib treatment. However, our non-clinical studies of imatinib transport used a panel of pharmacological inhibitors of several drug transporters, and many of these are not truly specific for individual transporters. Here we use cells stably transfected with hOCT1 to further characterise imatinib uptake. The BCR-ABL positive cell line KCL22 and the BCR-ABL negative human embryonic kidney line HEK293 were transfected with pcDNA3-hOCT1 (provided by Gründemann D, Cologne, Germany) with the empty vector pcDNA 3.1 used as a negative control. KCL22 was selected as it has low constitutive expression of hOCT1 in comparison to other BCR-ABL positive cell lines such as LAMA84, KY01 and K562. KCL22 and HEK293 cells were transfected using Nucleofector technology and Fugene 6 respectively. Positive clones were selected using the G418 antibiotic (Neomycin). Several stable clones for each line were obtained, expressing hOCT1 at different levels. These clones were then used for transport experiments, using cold and 14C radiolabelled pure imatinib (gift of Novartis). The influx of imatinib was greater in the stably transfected cells compared with the untransfected or cells containing the empty vector. Current data suggest that uptake is greater in clones with high hOCT1 expression than in those with lower expression. A time course assay also showed that uptake was very quick, but reached a plateau after 30 minutes. These findings support the view that hOCT1 is an important determinant of imatinib influx into BCR-ABL positive cells. Taken together with our earlier data on transporter expression on clinical samples, the present data underline the importance of baseline hOCT1 expression in determining the outcome of imatinib treatment in CML.

Molecular dignostics

0046

PROGNOSTIC SIGNIFICANCE OF MINIMAL RESIDUAL DISEASE MONITORING ON RELAPSE-FREE SURVIVAL IN CBFB-MYH11 ACUTE MYELOID LEUKEMIA

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Background. Detection of minimal residual disease (MRD) in acute myeloid leukemia (AML) associated with specific gene fusions is an important tool for the assessment of response to treatment and the individual risk of relapse. The real-time quantitative RT-PCR (RQ-PCR) method allows the quantification of fusion transcript levels at distinct time points during treatment. While in acute promyelocytic leukemia (APL) MRD monitoring has been clearly shown to be predictive for clinical outcome, the evaluation of the prognostic impact of MRD in CBFB-MYH11 AML remains difficult. Small patient populations and the availability of bone marrow/peripheral blood samples at defined time points mainly hamper most studies. *Aims.* To evaluate the prognostic impact of MRD in a large cohort of CBFB-MYH11 positive AML by RQ-PCR. *Methods.* A total of 44 patients (age 16-60 years) were treated within one of the AMLSG treatment trials (AMLHD93 n=4, AMLHD98A n=27, AMLSG 07-04 n=13). Patient samples (bone marrow and/or peripheral blood) were collected at study entry (n=75), during treatment course (n=176), as well as during follow up (n=110). All patients received two cycles of induction therapy with standard-dose cytarabine combined with etoposide and idarubicin. After a first high-dose cytarabine based consolidation therapy, patients received further high-dose cytarabine based consolidation (n=25), autologous transplantation (n=13) or allogeneic transplantation from a matched related family donor (n=6). Median follow up was 22.5 months. Evaluation of the quantitative CBFB-MYH11 fusion transcript expression for the three major fusion subtypes was performed by RQ-PCR using TaqMan technology. Primers were chosen according to Europe Against Cancer (EAC) standard protocols. Sensitivities ranged from 10^{-4} (type A, D) to 10^{-3} (type E). The fusion transcript copy number in each sample is reported as the normalized value of CBFB-MYH11 per 10^6 transcript copies of a housekeeping gene, β2-microglobulin, as a control. *Results.* Transcription levels at diagnoses ranged from 6208 to 312987 (median 34293.5). There was no prognostic impact of pretreatment transcript levels on relapse free survival. The ratio of transcription levels after two induction cycles and pretreatment levels ranged from 0 to 0.0049. Again, there was no prognostic impact of transcript levels after induction therapy. In contrast, during consolidation therapy 66% of the patients became RQ-PCR negative and RFS was significantly superior (RFS after 2 years 80%) compared to RQ-PCR positive patients (RFS after 2 years 35%) (p=0.02). After consolidation therapy, 5 of the RQ-PCR negative patients became positive at least in one sample during follow up. Three patients developed transcript levels above 10 and all relapsed, whereas in the two remaining patients only one sample during follow up became positive with transcription levels below 10. Both are in continuous remission. *Conclusions.* In our study, achievement of real-time quantitative RT-PCR negativity after consolidation therapy is significantly associated with favorable outcome in CBFB-MYH11 positive AML.

0047

CHARACTERIZATION OF THREE CASES OF ANEMIA/ MENTAL RETARDATION SYNDROMES (ATR-16) IN THE NETHERLANDS, USING MULTIPLEX LIGATION-DEPENDENT PROBE AMPLIFICATION (MLPA)

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Background. Two distinct and rare syndromes of a-thalassemia associated with mental retardation are known to date. One is characterized by the occurrence of large deletions involving the α-globin gene cluster on chromosome 16p (ATR-16 syndrome) and is most likely a contiguous gene syndrome. The other (ATR-X) involves mutations of the X-linked XNP gene, coding for helicase-2, a putative global transcriptional regulator. At present the molecular tests commonly used to identify deletion types of a-thalassemia and ATR-16 are gap-PCR, Southern blot or Fluorescent *in situ* Hybridization (FISH) analysis. However, the appli-

cability of these techniques is limited to known deletions, may involve radio-activity, is dependent upon the hybridization probes available and may require time consuming and laborious cell culture to generate metaphase chromosome spreads. *Aim.* Clinical description and molecular characterization of three independent patients presenting with a microcytic hypochromic anemia at normal ferritin levels and mild mental retardation. *Methods.* Multiplex Ligation dependent Probe Amplification (MLPA) is used to identify (unknown) deletions causing a-thalassemia, which remain undetected by the common techniques presently used in molecular diagnostics. *Results.* We have developed a subset of MLPA probes spread along a region of approximately 2Mb from the telomere of chromosome 16 up to the PKD-gene for high resolution mapping of rearrangements causing a-thalassemia. One Dutch Caucasian female (30 yrs) was identified because of a persistent microcytic hypochromic anemia at normal ferritin levels, positive I.B. test and no abnormalities at the molecular level using the standard detection *Methods.* The other two patients were identified because of (mild) mental retardation and the detection of a subtelomeric deletion by MAPH and mapped in detail by MLPA in the present study. The deletions causing ATR-16 in these patients vary in length between 1.5 to 1.9 Mb. *Conclusion.* We have developed a rapid and simple technique based on Multiplex Ligation-Dependent Probe Amplification for high resolution mapping of rearrangements involving the tip of the short arm of chromosome 16. Three cases show the rare ATR-16 syndrome, two of which were found by screening for subtelomeric imbalances by FISH or MLPA and not by hematological analysis. This would plead for more alertness when a patient presents with mild to moderate MR and microcytic hypochromic anemia with normal ferritin levels as suggestive for ATR-16.

0048

INDIVIDUAL PATIENT DATA ANALYSIS IN YOUNGER ADULTS WITH NORMAL KARYOTYPE AML: DIFFERENTIAL EFFECTS OF MOLECULAR MARKERS ON CLINICAL OUTCOME RESULTS OF THE AML STUDY GROUP

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Background. Mutations in the genes encoding NPM1, FLT3 (FLT3 ITD, FLT3 TKD), CEBPA, MLL (PTD) and NRAS have been identified as molecular markers in acute myeloid leukemia (AML) exhibiting a normal karyotype. Approximately 70% of normal karyotype AML have mutation in one of these genes. In most of the recent studies univariate analyses of outcome measures were performed for singles markers not taking into account potential interactions. Furthermore, little is known about the differential effect of different postremission therapies in the various genotypic subsets. *Aims.* To evaluate the prognostic impact of NPM1, FLT3 (FLT3 ITD, FLT3 TKD), CEBPA, MLL (PTD) and NRAS gene mutations on response to induction therapy and on survival probabilities following different postremission strategies [high-dose cytarabine-based chemotherapy (chemo), autologous (auto-SCT), or allogeneic stem cell transplantation (allo-SCT)]. *Methods.* Patients [16 to 60 years of age] were entered on four AMLSG treatment trials [AML-2/95, AML-1/99, AML HD93, AML HD98A]. Patients received two cycles of induction therapy with standard-dose cytarabine combined with etoposide and idarubicin. After a first consolidation therapy, in all four trials patients were assigned to allo-SCT if an HLA-identical sibling donor was available; in the AML-2/95 and AML HD93 trials, all other patients were assigned to chemo, whereas in the AML-1/99 and AML HD98A trials patients were randomized between chemo and auto-SCT. Diagnostic leukemia specimens were analyzed for mutations in the above genes. *Results.* Between 1993 and 2004, 872 patients exhibiting a normal karyotype were registered. Results of the mutation status were as follows (total number of samples analyzed; incidence of mutations): NPM1 (n=526; 53%), FLT3-ITD (n=516; 31%), FLT3-TKD (n=602; 11%), CEBPA (n=492; 16%), MLL-PTD (640; 7.3%), and NRAS (505; 13%). Complete remission rate (CR) was 77%. A logistic regression model identified the NPM1+/FLT3 ITD- (p<.0001) and the CEBPA+ genotype (p=0.03) as favorable prognostic factors for CR achievement. Cox proportional hazard models with limited backward selection for RFS and OS revealed age <48 years (hazard ratio (HR) 0.69 and 0.61), availability of an HLA-matched family donor (HR 0.58 and 0.73), the CEBPA+ (HR 0.42 and 0.36) and the NPM1+/FLT3 ITD- (HR 0.34 and 0.42) genotype as significant prognostic factors. There was no benefit for auto-SCT in any of the subgroups. The prognostic impact of an HLA-identical family donor varied significantly among the different molecular subgroups. *Conclusions.* Specific genotypes emerge as highly significant factors for response to induction therapy and for survival in patients with normal karyotype. The value of allo-SCT needs to be revisited in the various genotype subsets.

0049

DEVELOPMENT OF AN INTEGRATED ASSAY FOR QUANTITATIVE REAL TIME DETECTION OF BCR-ABL RNA FROM PERIPHERAL BLOOD

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Background. The current Europe Against Cancer Program practice guidelines for management of patients with CML call for the use of reverse transcription polymerase chain reaction (RT-PCR) assays during the initial workup of patients with chronic phase CML, in monitoring for minimal residual disease, and in identifying patients who may be at a high risk for relapse. *Aims.* RT-PCR testing for BCR-ABL is done today primarily using laboratory developed assays. While each of these assays may be consistent within any particular lab, there is still lack of complete consensus on assay design, results reporting, and reference ranges. Our goal was to develop a highly integrated assay that could be run easily by a laboratory technician without any special training in molecular techniques, and would give a standardized answer at any laboratory.

Table 1. Assay precision was assessed in a multi-center (3 sites), blinded, comparative study using four specimens with varying concentrations of RNA purified from the K562 leukemia cell line expressing the BCR-ABL transcript. Each site received a total of 80 specimens consisting of 20 specimens from each of the four different levels or RNA. Two technologists at each site partecipated in the 5 day study, each performing 2 runs of 4 specimens each per day. Each run included one specimen from each level. The percent ratio of BCR-ABL for each specimen was calculated using the delta Ct value generated by the GeneXpert. Precision was estimated in accordance with the NCCLS guideline for evaluation of precision performance of clinical chemistry devices. Cartridges that failed to produce a result were omitted from the data analysis.

Site	Sample	N	Ave% BCR-ABL/ABL	% BCR-ABL/ABL Std Dev	95% Confidence Interval	
1	Negative BCR-ABL	20	<0.000133% (negative)	*	*	*
2	Negative BCR-ABL	20	<0.000175% (negative)	*	*	*
3	Negative BCR-ABL	20	<0.000163% (negative)	*	*	*
Overall	Negative BCR-ABL	60	<0.000157% (negative)	*	*	*
1	Low BCR-ABL	20	<0.00477%	0.00477%	0.00218%	0.00737%
2	Low BCR-ABL	19	<0.00285%	0.00285%	0.00125%	0.00445%
3	Low BCR-ABL	20	<0.00528%	0.00528%	0.00241%	0.00815%
Overall	Low BCR-ABL	59	<0.00430%	0.00524%	0.00273%	0.00587%
1	Medium BCR-ABL	20	1.003%	0.324%	0.827%	1.179%
2	Medium BCR-ABL	20	0.856%	0.427%	0.624%	1.088%
3	Medium BCR-ABL	19	1.005%	0.433%	0.762%	1.248%
Overall	Medium BCR-ABL	59	0.954%	0.397%	0.835%	1.073%
1	High BCR-ABL	20	12.18%	2.40%	10.88%	13.49%
2	High BCR-ABL	20	12.34%	7.31%	8.36%	16.32%
3	High BCR-ABL	20	12.18%	4.10%	9.95%	14.41%
Overall	High BCR-ABL	60	12.24%	5.03%	10.74%	13.73%

- Negative samples (no added K562 RNA), all tested as negative, range = <0.0001% to <0.0009%
- Low positive samples (100 pg K562 RNA), 56/59 tested as positive, range = 0.0002% to 0.0207, 3/59 tested negaive, <0.0001%
- Medium positive samples (5 ng K562 RNA), all tested as positive, range = 0.2692% to 1.9817%
- High positive samples (100 ng K562 RNA), all tested as positive, range = 4.3414% to 36.8568%

Methods. The Cepheid Xpert® BCR-ABL Monitor assay is designed to co-amplify the BCR-ABL transcript and the ABL transcript (the endogenous control). The assay only requires a few simple manual pipetting

steps, followed by fully automated nucleic acid purification, nested RT-PCR, and data analysis. A 200 uL aliquot of whole blood is mixed with proteinase K and lysis reagent to inactivate nucleases and release the nucleic acid from the cells. After addition of 1 mL of ethanol to the lysed sample, the mixture is added to the test cartridge using a transfer pipette. Wash, rinse, and elution reagents are also added to designated ports in the test cartridge, the lid is closed, and the cartridge is loaded into the GeneXpert_ Dx System. By moving the sample and reagents into different chambers in the cartridge during the test process, the GeneXpert (1) isolates the total RNA from lysed whole blood by binding the RNA to the solid phase purification material, (2) washes and rinses away inhibitors, (3) elutes the RNA, (4) hydrates the reagent beads and combines the mixture with the eluted RNA, (5) moves the sample and reagent mixture into the reaction tube, (6) performs quality checks to ensure that reagent preparation was successful, (7) performs a one step RT-PCR followed by nested real-time PCR (8) reviews the signal from both the ABL endogenous control and the BCR-ABL transcript for acceptability, and (9) calculates the delta Ct between the two signals. The test process for BCR-ABL takes approximately 2 hours and 20 minutes. *Results and Conclusions.* The specificity of the assay was tested using 42 citrate and EDTA bloods from normal individuals and a collection of 12 bloods from patients with other hematologic disorders including acute myelogenous leukemia, acute lymphocytic leukemia, Hodgkin's lymphoma, multiple myeloma, and follicular lymphoma. All of these samples were negative for BCR-ABL, yielding a specificity of 100%. A collection of 46 samples from patients with CML were tested by the Xpert BCR-ABL Monitor assay and by a laboratory-developed RT-PCR reference assay. There was 85% agreement of negative results (17/20) and 100% agreement of positive results (26/26). A precision study indicated the assay was highly reproducible between sites, days, instruments, and operators (Table 1).

0050

HARMONIZATION OF BCR-ABL TRANSCRIPT QUANTIFICATION USING AN UNIFORM CONTROL PLASMID IN 37 INTERNATIONAL LABORATORIES

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Background. Serial measurement of leukemia specific BCR-ABL transcripts is a valuable approach to monitoring individual patients with chronic myelogenous leukemia after therapy. However, heterogeneity of molecular approaches results in a lack of comparability between different studies. Thus, there is an unmet need for harmonization of both procedures and expression of *Results.* In a series of consensus meetings within the European LeukemiaNet recommendations for achieving optimal sensitivity and standardization have been elaborated: (a) use of at least 10ml peripheral blood processed within 36 hrs; (b) bedside RNA stabilization for multicenter trials; (c) standardized PCR protocols optimized for each platform; (d) use of a single plasmid containing target and housekeeping genes to avoid dilution errors; (e) use of total ABL and/or β glucuronidase (GUS) as internal controls. *Aim.* The aim of the study was to assess the variability of results obtained from 37 different labs in 14 countries using the PAXgene Blood RNA Kit (PreAnalytiX, Hombrechtikon, Switzerland) for RNA extraction, individual protocols for cDNA synthesis, 3 different PCR platforms (TaqMan, TM, n=25, LightCycler, LC, n=13, Rotorgene n=1), and optimized quantitative RT-PCR conditions. *Methods.* In order to standardize results, b3a2 BCR-ABL and GUS sequences were cloned into a pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA), which was distributed to all participants in serial dilutions as external control for quantification of BCR-ABL, total ABL, and GUS. Ten samples containing dilutions (10, 2, 1, 0.1%) of b3a2 or b2a2 BCR-ABL positive in normal leukocytes and negative controls were prepared, blinded, and shipped to the participants. Transcript numbers were determined in triplicates, ratios BCR-ABL/ABL and BCR-ABL/GUS were calculated and expressed in percent. *Results.* Median ratios BCR-ABL/ABL for b3a2 samples were 9.1, 1.8, 0.85, and 0.11%; for b2a2 samples 9.5, 1.6, 0.84, and 0.11%. Median ratios BCR-ABL/GUS for b3a2 samples were 3.4, 0.77, 0.37, and 0.042%; for b2a2 samples 2.8, 0.48, 0.29, and 0.031%. Four of 37 participants (11%) detected low BCR-ABL copy numbers in negative control samples. The coefficients of variation (CV) for all participants, TM, and LC users were 0.62, 0.57, and 0.63 for ratios BCR-ABL/ABL; 1.03, 0.85, and 1.22 for ratios BCR-ABL/GUS, respectively. Standard errors to the regression line were significantly lower evaluating ratios BCR-ABL/GUS (median 0.075, range 0.0046-0.90) compared to ratios BCR-ABL/ABL (median 0.18, range 0.022-2.2, $p<0.001$). Overall, mean TM ratios were 1.7times higher than LC ratios indicating a difference of the amplification efficiency. *Conclusions.* Harmonization of BCR-ABL mRNA quantification is feasible employing a common plasmid for BCR-ABL, total ABL, and GUS. However, the remaining variability of results indicates minor differences of the PCR efficiencies using individual protocols. We therefore suggest the use of a common standard plasmid, the introduction of a calibrator, and regular control rounds to achieve comparability of results between individual labs.

0051

THE IDENTIFICATION OF JAK2V617F IN PATIENTS WITH POLYCYTHAEMIA IS HIGHLY CORRELATED WITH CONVENTIONAL CRITERIA FOR DIAGNOSIS OF POLYCYTHAEMIA VERA

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Background. In 2005 it was recognised that different myeloproliferative disorders (MPD) share an activating JAK2 tyrosine kinase mutation (JAK2V617F). The frequency of the mutation varies being greatest in polycythaemia vera (PV) (65-97%) and less common in essential thrombocythaemia and idiopathic myelofibrosis. *Aims.* We wished to assess the utility of screening for the JAK2V617F mutation in patients with known or suspected myeloproliferative diseases. In particular whether screening can contribute to the diagnosis or even substitute for other investigations in polycythaemia and if so what would be the economic implications. *Methods.* We adapted the screening assay for JAK2V617F described by Baxter[1] and over 6 months screened 145 consecutive patients with previously diagnosed or suspected MPD from different parts of New Zealand. Indications for screening were classified as polycythaemia, thrombocytosis, myelofibrosis and other. We undertook a retrospective review of the case records for those 66 patients who had been screened for polycythaemia, to ascertain which investigations had been performed at presentation, their results and information on subsequent management. Results of these investigations were used to establish or exclude a diagnosis of PV, using WHO or PVSG criteria. The frequency and cost of the various investigations performed for all patients were calculated. *Results.* The JAK2V617F mutation was detected in 31 of 66 (47%) patients with polycythaemia, 34 of 67 (51%) patients with thrombocytosis and 5 of 10 (50%) patients with myelofibrosis. Other patients screened included a patient with multiple myeloma who received an allograft from a donor with ET (JAK2V617F) and a patient with MDS and thrombocytosis (wild type allele). Of the 66 patients with polycythaemia, 12 patients were excluded since they had insufficient elevation of red cell mass, haemoglobin or haematocrit to meet criteria for PV. Of the remaining 54 patients, 42 patients had been sufficiently investigated to either diagnose or exclude PV. 24 of 25 (96%) patients with PV were JAK2V617F, whereas every one of 17 patients in whom PV was excluded had only the wild type allele. Twelve patients had been insufficiently investigated to determine whether or not they met criteria for PV - of these 5 were JAK2V617F. All patients with PV were receiving appropriate treatment with venesection or myelosuppresion. No patient in whom PV was excluded had received myelosuppressive therapy though many were on venesection programmes. 10200 EUR was spent on investigating 54 patients with polycythaemia. If JAK2V617F mutation screening had been undertaken as the initial investigation for these patients, proceeding to further investigations only in those without JAK2V617F, the total cost of investigations would have been 12400 EUR. *Summary/Conclusions.* In our patients with sufficiently elevated haemoglobin to meet WHO criteria for PV, the identification of JAK2V617F is 94% sensitive and 100% specific for PV. The varying frequencies of JAK2V617F reported in different series of PV patients may be due in part to the inclusion of patients in the PV group who do not meet diagnostic criteria for PV. Early screening for JAK2V617F in patients with polycythaemia can give a prompt and unequivocal diagnosis of PV without significant additional cost.

Reference

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0052

IDENTIFICATION OF A COMMONLY USED CDR3 REGION OF LGLS TCR ALPHABETA+/CD4+ VB 13.1 PATIENTS

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Background. Monoclonal TCRαβ+/CD4+/NKa+/CD8-/+dim T represent a subgroup of monoclonal LGL lymphoproliferative disorders different from both CD8+ T-LGL and NK-cell type LGL leukemias.The recently described TCRαβ+/CD4+ T-LGL leukemia/lymphocytosis has been shown to be associated in around one third of cases with a neoplasia other than the T-LGL, which prompted us to hypothesize that the TCRαβ+/CD4+ T-LGL may proliferate and expand as an effort of the immune system to control tumor growth, supporting in some way the antigen-driven selection model. We typed for HLA class I and II genes in patients with different TCR- Vβ expansions. TCR clonotypes and VDJ rearrangement structure were analyzed in a cohort of patients with CD4T-LGL expansions. *Aims.* Analyse the possible association between the TCR Vβ family expanded with HLA and CDR3 hypervariable region expressed in patients with Vβ expansions LGG TCR αβ+/CD4+ /CD8±d. *Methods.* A total of 36 individuals (19 males and 17 females; mean age of 64±11 years, ranging from 40 to 81 years) having a TCRαβ+/CD4+/NKa+/CD8-/+dim monoclonal T-LGL lymphoproliferative disorder were studied. For the immunophenotypic studies a panel of 24 monoclonal antibodies (MAb) directed against an identical number of members of 21 different TCR-Vβ families was used. A genotyping for HLA-ABC and both HLA-DRB1 and HLA-DQB1 were performed by SSPO-PCR. DNA were amplified and clonal products from the VH gene PCR were sequenced directly using the BigDye Terminator Cycle Sequencing Reaction Kit. *Results.* In all cases studied, expanded CD4+ LGL T-cells showed relatively high SSC features as compared to normal PB CD4+ T-lymphocytes and common phenotypic characteristics, consisting of TCRαβ+/CD4+/CD8-/+dim cells with a typical cytotoxic (granzyme B+, CD56+, CD57+, CD11b±) activated/memory T-cell immunophenotype (CD2+^{bright}, CD7-/+d, CD11a+^{bright}, CD28-, CD62L-, HLA-DR+). Flow cytometric analysis of the TCR-Vβ repertoire of CD4+/CD8-/+dim LGL T-cells was consistent with a (mono)clonal expansion in all cases studied, which accounted for 75%±26% of all PB CD4+ T-cells. In 27 cases the expanded TCR-Vβ family was identified with the panel of TCR-Vβ reagents used, corresponding to TCR-Vβ13.1 in 15 cases (42%), TCR-Vβ 2.1 in 2 (5.6%), TCR Vβ 3.1 in 2 (5.6%), TCR-Vβ 8.1 and Vβ 8.2 in 2 (5.6%), TCR-Vβ17.1 in 2 (5.6%), TCR-Vβ 22 in 2 (5.6%) and TCR-Vβ11 or TCR-Vβ14.1 in one case each (2.8%). In the remaining 9 patients, the expanded TCR Vβ family was not identified (25%) with the panel of MAb used. All 15 patients who showed expansions of TCRVβ 13.1+ CD4+ T cells were HLA-DRB*0701+.Comparison of CDR3 size distribution in clonal CD4+/CD8-/+dimT-cells from the same patients showed a highly restricted usage of VHDJH segments and shared CDR3 configurations. *Conclusions.* These findings suggest that the expansions were selected for this unique TCR structure. These results strongly suggest that Vβ13.1 CD4+ T cells with the described CDR3 motif may recognize a specific antigen presented by DR7 molecules, indicating the existence of a common associated antigen.

0053

MDR1, MRP AND LRP EXPRESSION IN PATIENTS WITH UNTREATED ACUTE LEUKEMIA: CORRELATION WITH TC-99M MIBI BONE MARROW SCINTIGRAPHY

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Background. Multidrug-resistance (MDR) phenotype concerns altered membrane transport that results in lower cell concentrations of cytotoxic drug in many cancer types, including leukemia and is related to the overexpression of a variety of proteins that act as ATP dependent extrusion pumps. Tc-99m Sestamibi (MIBI) is a transport substrate for Pgp pump. *Aim.*We assessed the bone marrow uptake of Tc-99m MIBI and its correlation with messenger RNA (mRNA) levels of MDR1, Multidrug-Resistance Associated protein (MRP) and Lung Resistance Protein (LRP) in acute leukaemia. *Methods.* A total of 26 patients with new diagnosed acute leukaemia (8 ALL and 18 ANLL) were included the in the study. The expression of MDR1, MRP, and LRP on mRNA levels were assessed by semi quantitative RT-PCR (Roche Light Cycler System, Metis Biotechnology primers and probes for MDR1, MRP and LRP) in the blast cells from the bone marrow samples. Planar images of the pelvis and thorax were acquired 20 min after injection of 740 MBq Tc-99m MIBI. The MIBI uptake in the bone marrow was evaluated using a quantitative scoring system with determination of the tumour-to-background ratios for the bone marrow in areas that included the proximal femur, anterior iliac crest and sternum. The correlation between the RT-PCR results and MIBI uptakes was analysed by using Spearman's rank correlation coefficients with two-tailed test of significance. *Results.* There was an inverse relationship between Tc-99m MIBI uptake of bone marrow and both mRNA levels of MDR1 and MRP (p=0.000, r= - 733 and p=0.001, r= - 610, respectively). No correlation was found between MIBI uptake and mRNA levels of LRP. Conclusion: Increased expression of MDR1 and MRP correlates with a low accumulation of Tc-99m MIBI in bone marrow areas in patients with acute leukaemia. As a functional imaging, Tc-99m MIBI bone marrow scintigraphy can identify the MDR1 and MRP phenotype, but not LRP, in patients with acute leukaemia.

0054

REAL-TIME RT-PCR ASSAY USING THE TAQMAN PROBES WITH LNA (LOCKED NUCLEIC ACID) MODIFICATION TO DETERMINE JAK2 GENE V617F MUTATIONS IN MYELOPROLIFERATIVE DISEASES (MPD)

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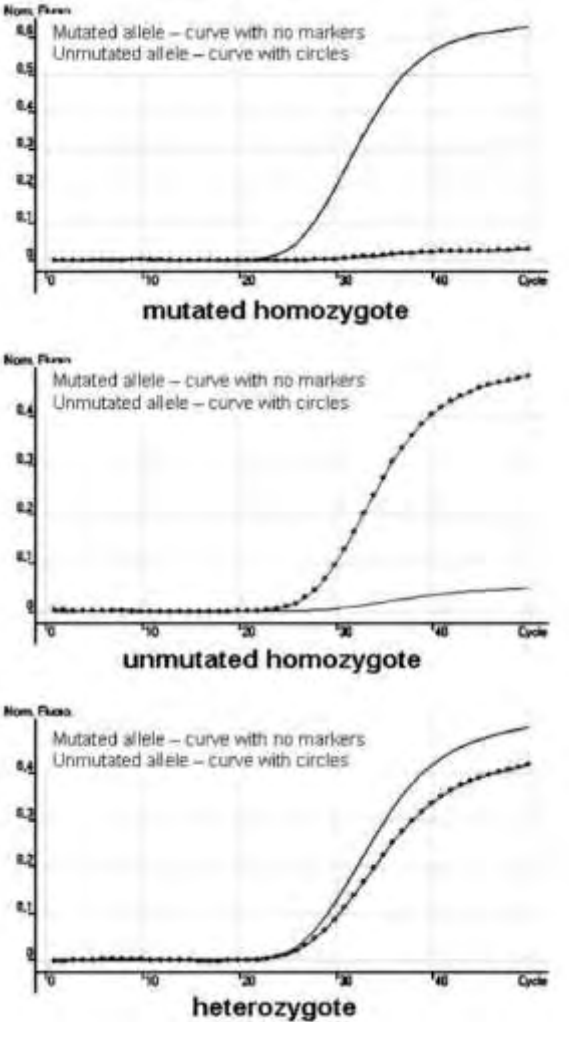
Background. JAK2 V617F mutations are frequently found in MPDs. JAK2 mutations confirm clonality in MPD, and according to some authors, they may be relevant even prognostically. In the near future, therapy by JAK2 inhibitors may be foreseen. The *classical* method of detection of JAK2 V617F mutations published by Baxter *et al.*[1] takes advantage of mutation-specific primers in PCR and requires sequencing to distinguish between homo- and heterozygous mutations. *Aims.* We have developed a more straightforward, real-time RT-PCR method, to demonstrate JAK2 mutations, allowing zygosicity discrimination.



Methods. Peripheral blood granulocytes were separated from altogether 151 patients with already diagnosed or suspected Ph- MPD. Patients with polycythaemia vera (PV), secondary polyglobulia (SP), essential thrombocythemia (ET), idiopathic myelofibrosis (IMF) and undifferentiated MPD (MPD-U) were included in the study. The cells were lyzed and RNA extracted using the Trizol reagent. Following reverse transcription, two methods were employed to detect JAK2 mutations. 1) The method according to Baxter *et al.*,[1] using two forward primers, one of them hybridizing to the mutated allele and a common reverse primer recognizing both the mutated and unmutated JAK2 alleles. Homo- and heterozygosicity of the mutated gene was discriminated by sequencing analysis. 2) The allelic discrimination real-time RT-PCR assay that uses one pair of primers and two dual labeled TaqMan probes with LNA modified nucleotides. The probes differ at the polymorphic site, one of them is complementary to the wild-type JAK2 allele and the other to the mutated one. The result is given by the curves arising from measured fluorescence of two different reporter dyes during the real-time PCR (FigURE 1).*Results.* Altogether 151 samples of patients with suspected Ph-MPD were analyzed using both of the above mentioned methods for JAK2 detection. In both of the assays, the same result was obtained, JAK2 mutation being found in the same 71 out of 151 patients (47.0%). Ten of the 71 JAK2 mutations (14.1%) were homozygous, half of which were found in PV patients. In ET, JAK2 mutations were demonstrated in 22/57 (38.6%) patients, none of them was homozygous. Of 43 patients with PV, 33 had mutations (76.7%), whereas only 1/10 patients with SP had the mutated allele of JAK2 gene. Six of 20 (30.0%) individuals with IMF had JAK2 mutations (3 were homozygous). In the remaining 21 MPD-U patients, 9 mutations (42.9%) were detected. *Conclusions.* The TaqMan allelic discrimination assay yields the same results as the method of Baxter *et al.*[1] In contrast to the latter, it is very simple and does not require sequencing to distinguish between homo- and heterozygotes. Thus it is less laborious and time-consuming and therefore also suitable for routine clinical laboratory testing.

References

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0055

HIGH FREQUENCY OF AML1 MUTATIONS IN BOTH *DE NOVO* MYELODYSPLASTIC SYNDROME AND CHRONIC MYELOMONOCYTIC LEUKEMIA BUT WITH DIFFERENT MUTATION PATTERNS

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Background. Transcription factor AML1 is essential for normal hematopoiesis. AML1 mutations have been found in therapy-related myelodysplastic syndrome (MDS) but were rarely described in patients with *de novo* MDS or chronic myelomonocytic leukemia (CMML). *Aims.* We sought to determine the frequency and patterns of AML1 mutations in *de novo* MDS and CMML and to correlate the mutation status with the clinicohematologic features. *Methods.* Mutation analysis of AML1 was performed on bone marrow samples from 76 patients with MDS (11 RCMD, 31 RAEB1 and 34 RAEB2) and 67 patients with CMML by direct sequencing for all RT-PCR products amplified with 3 overlapping primer pairs which cover the coding sequences of AML1 gene from exon 3 through exon 8. *Results.* At initial presentation of MDS, 14 of 76 MDS patients (18.4%) had AML1 mutations; 3 mutations were located in Runt homologogy domain (RHD) (exons 3-5) whereas 11 mutations were located in the non-RHD region (exons 6-8). The 14 AML1 mutations included 6 missense mutations, 4 nonsense mutations, 2 frameshift mutations, and 2 silent mutations. AML1 mutations were detected in 27 of 67 CMML patients (40%) at initial diagnosis, 17 patients had 19 mutations located in RHD and 10 patients had mutations located in the non-RHD region; the patterns of 29 mutations consisted of 7 missense mutations, 5 nonsense mutations, 14 frameshift mutations and 3 silent mutations. One CMML patient had two missense mutations in RHD, another patient had two frameshift mutations in RHD. Cloning analysis showed that the two mutations were on different alleles in both patients. The frequency of AML1 mutations was significantly higher in patients with CMML than in MDS (p=0.005). Mutations in RHD occurred more frequently in CMML than in MDS (p=0.020). CMML patients had a higher frequency of frameshift mutations as compared with MDS patients (p=0.045). AML1$^+$ CMML patients had a significantly lower platelet count than AML1$^-$ patients (p=0.025). There were no differences in age, sex, hemoglobin level, WBC count, percentages of blasts in bone marrow and peripheral blood, morphologic subtype, and cytogenetic risk group between AML1$^+$ and AML1$^-$ patients in CMML or MDS. Eleven of 14 AML1$^+$ MDS patients (78.6%) progressed to AML compared with 39 of 62 AML1$^-$ patients (62.9%) (p=0.357). Eleven of 27 AML1$^+$ CMML patients (40.7%) progressed to AML compared to 13 of 40 AML1$^-$ patients (32.5%) (p=0.605). Time to AML transformation and overall survival of AML1$^+$ patients did not differ from AML1$^-$ patients in both MDS and CMML groups. *Conclusions.* Our study showed that AML1 mutations were frequently detected in *de novo* MDS and CMML, especially the latter. Patients with CMML were more frequently associated with mutations in RHD and frameshift patterns compared to patients with *de novo* MDS.

0056

NPM1 AND FLT3 MUTATIONS, DUPLICATIONS OF MLL AND EXPRESSION OF GENES WT1, EVI AND BAALC AS PROGNOSTIC FACTORS IN PATIENTS WITH *DE NOVO* ACUTE MYELOID LEUKEMIA

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The cytogenetic analysis allows to classify the AML in different risk groups, nevertheless in about 50% of AML patients carry normal caryotype by conventional cytogeneticts and they lack of prognostic markers. Recently, several molecular alterations have been related to AML. This study analyze the prognostic impact of FLT3 mutations (ITD and D835 mutations) NPM1 mutations, partial tandem duplications (PTD) of MLL as well as WT1, EVI1 and BAALC gene expression in a group of 100 adult patients (48 female and 52 male; median age 62 years, range 22-94) with AML *de novo*. Screening for NPM1 was performed using a melting curve assay based Lightcycler (Schnittger S *et al.* Blood 2005) and confirmed by direct sequencing in ABI 310. The presence of FLT3 ITD was detected according to the method of Nakao M *et al.* (Leukemia 1997) and D835 using a melting curve based Lightcycler assay designed by Tib Molbiol (Berlin, Germany). MLL PTD was analyzed according to the method of Caligiuri MA et at. (Cancer research 1996). Gene expression quantification for WT1, EVI1 and BAALC was performed by real-time PCR ABI Prism using β-glucuronidase (GUS) as control gene and TaqManTM probes technology. Frequency of mutations: NPM1 mutations were found in 25/82 patients (30.5%) and four different mutations were detected: type A (72%), B (8%), D (12%), Km (8%), FLT3 mutations were present in 16/97patients (16.5%) (12 ITD and 4 D835) and the incidence of mutation for MLL PTD was of 4/74 patients (5.4%). Gene Expression: WT1, EVI1 and BAALC showed a median gene expression ratio: 0.28 (range 0-7.03), 0.013 (range 0-3.35) and 0.01 (range 0-18.90) respectively. Overexpression criteria was defined according to the median expression ratio Clinical characteristics: FLT3 and NPM1 mutations were significantly associated with a high white blood cell count (WBC) (p=0.003 and p=0.002 respectively). In addition, NPM1 mutated cases were significantly associated with FLT3 mutations (p< 0.0001), normal karyotype (p=0.024), and the monocytic lineage (FAB M4/M5, p=0,036). Prognostic impact: The response to induction showed no relation with any of the molecular markers. The disease -free survival (DFS) was significant influenced by overexpression of WT1 (p=0.045), FLT3 mutations (p=0.037), a high WBC (p= 0.047) and the cytogenetic risk group (p= 0.0034). In conclusion, our data show that the analysis of WT1 expression accompanying the FLT3 of mutations may be useful to predict prognosis beside cytogenetic findings. This study partially has been supported by grant FIS03/0400.

0057

ACCURATE V617F JANUS KINASE 2 MUTATION GENOTYPING COMBINING ARMS PCR AND CAPILLARY ELECTROPHORESIS

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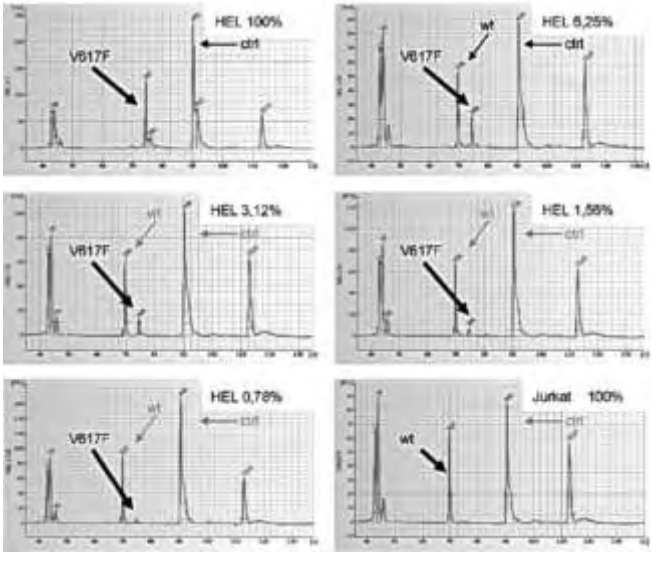
Background. During the last decade CML has paved the way for tyrosine kinase-targeted therapy. Recently, several groups have demonstrated the pivotal role of the acquired V617F Janus kinase 2 (JAK2) mutation in Ph- Chronic Myeloproliferative Disorders, indicating a novel potential therapeutical target in CMPDs. Although this mutation has been described in the major CMPDs subtypes, controversy still remains about its exact prevalence in each subcategory. This could partially indicate the difficulties of achieving a precise diagnosis relying on the current WHO and PVSG diagnosis criteria. On the other hand, different technologies characterized by various sensitivity and sensibility have been used in the princeps studies, highlighting the need for standardized, accurate and sensitive assays. *Aims.* We describe here a new V617F JAK2 mutation screening approach. The analytical properties of this assay are described and compared with the traditional PCR sequencing strategy, site-specific restriction analysis and ARMS PCR followed by slab-gel electrophoresis. Finally, we report our experience in genotyping 80 controls and 222 patients sent to our lab for typical and atypical MPD diagnosis. Clinical parameters, as well as previously described clonality assay, i.e. granulocytes PRV-1 expression, were evaluated in regard of the JAK2 V617F genotype. *Methods.* We developed a new V617F JAK2 mutation screening assay which combined the previously described amplification refractory mutation system (ARMS) and capillary electrophoresis performed with the Agilent 2100 Bioanalyzer apparatus (ARMS-cap). Serial dilutions of 100% V617F-homozygous HEL cell line into non mutated Jurkat cell line were assessed by ARMS PCR, ARMS-cap, PCR sequencing and BsaXI site-specific restriction analysis. The sensitivities of these tools were compared.



Results. ARMS PCR followed by slab-gel electrophoresis presented a JAK2 V617F detection sensitivity ranging from 1/32 (DNA) to 1/256 (RNA), which is far better than the PCR sequencing resolution (1/8 to 1/16). BsaXI cleavage improved the sensitivity when starting from DNA (1/64-1/128) whereas its best sensitivity level was the same as ARMS RNA PCR (1/256). ARMS-cap, offering a resolution of 1/64 (DNA) and 1/512 (RNA), improved the sequencing approach as well as the ARMS PCR followed by slab-gel electrophoresis. These results were in the same range than the BsaXI cleavage assay (1/128-1/256). Using this tool to assess our cohort of patients, we found the following incidence of JAK2 V617F mutation: PV, 90% (18/20); TE, 44% (12/27); IMF, 80% (4/5) and aCMPD, 24% (16/66). None of the 10 ALL; 10 *de novo* AML, 14 primary AML, 10 NHL, 10 HES/CEL, 25 MDS, 11 CMML were found to be mutated whereas we found 1 CNL and 4% MDS (1/25) harboured the mutation. *Conclusions.* JAK2 ARMS PCR assay combined with capillary electrophoresis represents a new and sensitive assay for an accurate V617F JAK2 mutation screening. The analytical properties of this assay overcome the classical PCR-sequencing or ARMS PCR followed by slab-gel electrophoresis approaches. Whereas this approach only slighltly improve the raw sensitivity level achieve by BsaXI site-specific restriction, in silico electrophoresis followed by automated data collection and recording offers an objective and reproducible tool for V617F JAK2 mutation screening.

0058

COOPERATING MUTATIONS OF RECEPTOR TYROSINE KINASES AND RAS SIGNALING PATHWAY IN CHILDHOOD CORE-BINDING FACTOR AML WITH EMPHASIS ON C-KIT MUTATION AND A COMPARATIVE STUDY BETWEEN DIAGNOSIS AND RELAPSE

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Background. There have been a few studies on activating mutations in Ras and receptor tyrosine kinases (RTK) such as c-KIT and FLT3 in pediatric acute myeloid leukemia (AML) or pediatric core-binding factor (CBF) AML. We examined the cooperating mutations of RTK and Ras signaling pathway in childhood CBF AML with emphasis on c-KIT mutation and a comparative analysis between diagnosis and relapse. *Methods.* Among the 151 childhood AML patients, thirty-eight were identified to have CBF AML including 27 AML1-ETO and 11 CBFβ-MYH11. Bone marrow samples were analyzed for c-KIT, FLT3-ITD, FLT3-TKD, N-Ras and K-Ras mutations. Mutation analysis of c-KIT was performed by direct sequencing for all cDNA PCR products amplified with 5 overlapping primer pairs which cover the whole coding sequences of c-KIT gene from exon 1 through exon 21. *Results.* The frequencies of c-KIT, FLT3-ITD, FLT3-TKD, N-Ras and K-Ras mutations were 41% (15/37), 0% (0/38), 3% (1/36), 8% (3/38) and 5% (2/38), respectively. Together, 53%(20/38) of childhood CBF AML had a collaboration with RTK/Ras mutations. All these mutations were mutually exclusive but one patient who had both c-KIT and N-Ras mutations. Of the 15 patients with c-KIT mutations, 4 had single mutations in exon 8: T417_D419delinsF, Y418_D419delinsG, T417_R420delinsRGK, and D419del. Eight had single mutations in exon 17: D816Y in 3 patients, N822K in 3 patients, D816H and D816V in one each. Three patients had two c-KIT mutations: one each with [Y418_D419del$^+$ D816H], [Y418N$^+$Y418_D419insFF], and [D816Y$^+$ N822K]. Cloning analysis showed that the combined mutations were on the same alleles or on different alleles. For patients with c-KIT mutations, the mutations were absent in all the complete remission samples examined. Six patients relapsed, 4 of them had c-KIT mutations at diagnosis, all 4 relapsed with identical c-KIT mutations as initial diagnosis and none gained or lost mutations. The 5-year overall survival (OS) of CBF AML patients was 70±8.1% (s.e.) and the 5-year event-free survival (EFS) was 68±8.0%. The 5-year OS of c-KIT$^+$ and c-KIT$^-$ patients were 54±14.1% and 81±8.8%, respectively (p=0.191). The 5-year EFS were 56±13.7% for c-KIT$^+$ and 76±9.5% for c-KIT(-) patients (p=0.323). The 5-year OS for those carrying any one of RTK/Ras mutations was 60±11.9% compared with 81±10.0% for those without mutation (p=0.265); and the 5-year EFS was 61±11.7% for mutation$^+$ compared with 75±10.9% for mutation$^-$ patients (p=0.444). *Conclusions.* Our study showed that 53% of childhood CBF AML had RTK/Ras mutaions, with c-KIT being the most common (41%). Patients carrying c-KIT mutations relapsed with the identical patterns indicating that c-KIT mutations play a crucial role in the leukemogenesis in a subset of CBF AML.

0059

SENSITIVE DETECTION OF C-KIT POINT MUTATIONS IN PATIENTS WITH MASTOCYTOSIS BY D-HPLC

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Background. The majority of patients with systemic mastocytosis (SM) are associated with an activating mutation in codon 816 of c-kit (CD117), a tyrosine kinase receptor on the surface of mast cells. This abnormality is regarded as being causative for the pathogenesis of the disease and as a potential target for therapeutic intervention. The sensitivity of screening procedures for mutations by direct sequencing might be compromised by a small proportion of malignant cells in the bone marrow (BM) sample. Therefore sensitive methods are required for diagnosis and surveillance of pts during therapy. Imatinib inhibits the c-Kit tyrosine kinase at pharmacological doses with an IC50 of 0,1 µM, but it does not affect D816V mutants. Recent *in vitro* data suggest that both dasatinib, nilotinib (AMN107) and midostaurin (PKC412) have inhibitory effects on c-Kit D816V mutant cells. *Aim.*We sought to set up a sensitive strategy to detect c-kit mutations in BM and peripheral blood (PB) samples using D-HPLC (denaturing-high performance liquid chromatography) combined with direct sequencing. *Methods.* D-HPLC has been established using serial dilutions of D816V c-kit positive HMC-1 cells in a background of NB4 cells harboring wildtype c-kit. The technique was then applied to 79 pts fulfilling the WHO criteria for SM. In case of a positive D-HPLC signal, c-kit exon 17 was sequenced to confirm the mutation using D-HPLC eluates and/or cDNA from the original sample. *Results.* D-HPLC was optimized to detect down to 0.1-0.5% HMC-1 cells. In comparison, the detection limit for D816V point mutations by conventional sequencing was 10%. BM (n=79) and/or PB (n=7) samples from 77 pts (42 m, 35 f) have been investigated. Median age was 51 yrs (range 23-81). At diagnosis, D-HPLC was positive in BM samples from 70/79 cases (89%), conventional sequencing revealed the D816V mutation in 56 pts, one pt was positive for the D816H mutation. In addition to D816V, an I798I polymorphism was observed in one pt. The analysis of PB only revealed D-HPLC positivity in 5/7 pts with a consecutive detection of a D816V mutation in three pts. *Conclusions.* (i) D-HPLC combined with conventional sequencing is a reliable and sensitive method to detect c-kit mutations in the majority of pts with SM. (ii) The method is eligible for the surveillance of pts during therapy with novel tyrosine kinase inhibitors.

0060

MUTATIONAL SCREENING IN A POPULATION OF HAEMOPHILIA A SUBJECTS FOLLOWED AT THE HAEMOPHILIA CENTRE IN CENTRO HOSPITALAR DE COIMBRA

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Introduction. Haemophilia A (HA) is an X-linked hemorrhagic disorder associated with blood coagulation factor VIII (FVIII) deficiency. FVIII gene mutations type are closely correlated with the FVIII activity levels, however, the same mutation can generate different phenotypes and not all the haemophilia patients with FVIII levels <1% bleed with the same intensity and frequency. *Aim.*In order to provide the HA carriers identification and eventual prenatal diagnosis, we performed the FVIII gene molecular studies in the HA patients followed at our Haemophilia Centre. In this study we present the mutations found, their correlation with the severity of the disease and the development of FVIII inhibitors. In the severe haemophiliacs group we screened the Factor V Leiden (FVL) and prothrombin G20210A variant (PRT G20210A) to evaluate their role as phenotype modulating factors. *Material and Methods.* 53 proposita with HA, and 11 affected relatives, classified as severe (32), moderate (5) and mild (16) according to ISTH criteria. FVIII gene molecular studies: intron 22 and intron 1 rearrangements were studied by PCR techniques; other mutations were screened by direct sequencing of PCR fragments spanning the promoter region, all the exons and the intron-exon boundaries. The FVL and PRT G20210A were screened by Multiplex Allele-specific PCR. *Results.* Twenty nine different mutations were identified in 52/53 patients, 15 of which have not been previously reported in HAMSTeRS. In the group of severe HA patients (n = 32) seventeen (53%) have the IVS22 inversion, two (6%) have the IVS1 inversion, 1 has a missense mutation, 2 have splicing mutations, 4 have small deletions, 2 have large deletions and 3 have insertions. In one patient we are still looking for the mutation. Missense mutations were the most common (81%) among the moderate and mild HA patients (n=21). In this group we found a frameshift mutation, due to the small deletion c.3637delA p.I1194fsX4 in exon 14, in one haemophiliac with cFVIII=19%. Five out of 35 severe HA patients developed FVIII inhibitors: two, high responders, carry the IVS22 inversion (2/14), 2 have the IVS1 inversion (2/3) and 1 has a large deletion (1/2). One mild haemophiliac with a missense mutation developed inhibitors and is a low responder. Four severe HA carried prothrombotic risk factors (4/35): 2 have FVL (one is homozygous) and 2 have the PRT G20210A. *Conclusions.* In our HA patients, the severe phenotype is mostly associated with gene rearrangements (IVS22 and IVS1) (19/32). Six out of 7 frameshifts identified are responsible for premature stop codons. The exception is the deletion c.3637delA p.I1194fsX4, associated with a mild phenotype, in which a reading frame correction probably occurs at the mRNA level. In the moderate and mild phenotypes the majority of mutations identified are missense transitional mutations. The mild haemophiliac who developed inhibitors after treatment for surgery, has a mutation near an antigenic determinant region of FVIII. Amelioration of the phenotype is evident in the patient homozygous for FVL, as he only needs 2-3 treatments per year and his first hemorrhagic episode was traumatic, at the age of 3.

Table 1. New mutations identified at the Haemophilia Centre in CHC.

	Exon/Intron	*Nucleotide Exchange*	*AASubstitution*	*Domain*	*Severity*
	3	nt 269, T>C	Leu 71 Pro	A1	Severe
	3	nt 343, G>T	Val 96 Phe	A1	Moderate
	9	nt 1347, A>G	Tyr 431 Arg	A2	Mild
	11	nt 1589, A>G	Tyr 511 Cys	A2	Mild
Missense	12	nt 1858, G>A	Val 601 Met	A2	Mild
	13	nt 2045, T>C	Val 663 Ala	A2	Mild
	17	nt 5738, A>G	Asn 1894 Ser	A3	Mild
	25	nt 6793, C>A	Gly 2246 Lys	C2	Mild
	26	nt 6954, C>G	Pro 2300 Arg	C2	Moderate
Splicing	Intron 7 (acceptor)	ag/ATG>cg/ATG	IVS7-2	A1	Severe
	Promotor-3	-	-	A1	Severe
Deletions	18 (inframe)	3 nt (5962-5964 del GAG)		A3	Mild
	21-22	-	-	C1	Severe
Insertion	1	1 T at codon 24	Frames hift	A1	Severe
	14	Duplic 13 bp (cd 836-840)	Frames hift	B	Severe

nt: nucleotide; del: deletion; duplic.: duplication; bp: base pair; cd: codons).

0061

MOLECULAR CLINICAL CORRELATION OF G6PD DEFICIENCY IN WESTERN SAUDI ARABIA

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Background. High frequencies of Glucose-6-Phosphate Dehydrogenase (G6PD) deficiency have been reported in most countries in the region. In Saudi Arabia, G6PD deficiency exists at variable frequency in different provinces of the country. G6PD deficiency may cause neonatal jaundice, sepsis, hemolytic anemia (favism) following consumption of broad beans, and stress oxidative hemolysis occasionally can cause severe hemolytic anemia following treatment with specific drugs or participated by infection. *Aims.* The aim of this study was to investigate the mutation spectrum and clinical significance of the G6PD gene among population in western Saudi Arabia. *Methods.* A total of 492 unrelated native Saudi volunteers of both sexes (224 male, 268 female) were screened for G6PD deficiency by quantitative *Methods.* DNA was extracted from 42 G6PD-deficient Saudi subjects (36 males and 6 female). These subjects were screened for gene mutations using polymerase chain reaction/restriction fragment length polymorphism (PCR-RFLP). Screening included Mediterranean563C T, and Aures143T C, and A_202G A, 376A G. *Results.* G6PD Mediterranean mutation 563C T accounts for most cases of G6PD deficiency in Saudi nationals followed by G6PD Aures143T C representing 38% and 17%, respectively. A new polymorphic variant (17%) has been identified during the course of this study although none of the samples showed A- mutation. Overall fre-

quency of G6PD deficiency is 0.265. G6PD Aureus showed more severe clinical manifestation. *Summary/Conclusions.* This study has characterized the molecular heterogeneity of G6PD variants among Saudis in the western Saudi Arabia suggesting significant gene flow. G6PD quantitative method and molecular characterization of G6PD deficiency shows high correlation with clinical manifestation.

0062

PREVALENCE OF NMP1 MUTATION IN AML AND MDS

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Background. A mutation on the nucleophosmin gene (NMP1) has been recently described on 25-35% of all AML patients. NMP1 is located on the 5q35 chromosomal region and this mutation, found on exon 12 of the NMP1 gene, causes the translocation of the NMP1 protein to the cytoplasm. This mutation is also linked to a wide spectrum of morphologic AML subtypes, normal karyotype, a better response to induction chemotherapy, and a higher prevalence of FLT3-ITD. *Aims.* To analyse the prevalence and prognostic value of the NMP1 mutation on AML and MDS patients. *Methods.* A total of 55 patients with non promyelocytic AML were studied. These patients were previously examined for the FLT3-ITD mutation (5 M0, 8 M1, 10 M2, 12 M4, 4 M5, 1M6 and 15 not determined according to FAB criteria) and 12 MDS. The average age of the patients ranged from 18 to 95 years. Screening for NMP1 mutation was undertaken by the LightCycler system according to the Schnittger et al technique (Blood 2005). *Results.* The NMP1 mutation was detected in 30.9% of patients with AML (15 out of 55), being this prevalence higher than that found for the FLT3-ITD, in the same population (23.8%). The 17 NMP1+ AML distributed as follows : 8 M1-M2 (44%), 4 M4-M5 (25%), and 5 not determined (33.3%). 68% of the NMP1+ patients had a normal karyotype, while 9% of them had cytogenetic anomalies. The FLT3-ITD mutation was found in 41.2% of the NMP1+ AML cases. The global mortality was analyzed with disregard to any risk factors, with a mortality of 67% in the NMP1+/FLT3-ITD- group standing in clear contrast to a 100% death rate in the NMP1+/FLT3-ITD+ group. Within the limits of the group studied, no sifginicant prevalanece of the NMP1 mutation was observed regarding the sex of the patient. *Conclusions.* 1) The prevalence of the NMP1 mutation in our LMA group was 30.9%, and contrary to what has been described in literature, a higher incidence on M4 and M5 subtypes was not found. As recently published by Thiede C *et al.,* a higher incidence on M1 and M2 subtypes was found. 2) The NMP1 mutation prevalence was high on MDS patients, which to our knowledge, had not been previously described in literature. 3) The other genetic anomaly most commonly associated to the NMP1 mutation was FLT3-ITD. 4) The screening for this mutation could be useful in the future when grouping patients with normal karyotype in a subgroup with better prognostic. 5) The high incidence of the NMP1 mutation in patients with MDS could suggest a role for this gene in the pathogenesis of this disease.

0063

THE COMPARISON OF THE RESULTS OF MOLECULAR MONITORING OF IMATINIB THERAPY IN CML BCR-ABL POSITIVE PATIENTS USING QPCR AND TWO CONTROL REFERENCE GENES

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Background. The tyrosine kinase inhibitor imatinib mesylate (Gleevec, STI571) has proven to be an effective new therapy for patients with chronic myeloid leukemia (CML). Quantitative reverse transcription-polymerase chain reaction (qPCR) for detection of BCR-ABL transcripts is frequently used for monitoring patients. In available publications there is still no homogenous and precision recommendations for standardization of the detection of BCR-ABL transcripts. The main doubts concern choice of qPCR machine and control gene. The ABL gene is definitely recommended, but other genes (like BCR, GUS*p*= β-glucuronidase, β-2-microglobulin, G6PD etc) are also acceptable. *Aims.* in this study we compared the usefulness of the qPCR methods using two control reference genes (GAPDH and cABL) for monitoring of effectiveness of imatinib therapy. *Methods.* the study group consisted of 33 patients (16 female, 17 male) with confirmed diagnosis of CML (32 in chronic phase and 1 in accelerated phase). 10ml peripferal blood was taken every 3 months. Total cellular RNA was obtained by phenol-chloroform extraction, isopropanol precipitation and washing with 700 mM/L ethanol. Samples were stored in temperature up do -80°C. The level of BCR-ABL transcripts was measured in Rotor Gene 2000 machine (Corbett Research). The amounts of BCR-ABL were also calculated from a 8-well ready-to-use reference dna strip containing 8 defined amounts of BCR-ABL as a quantification control in a range of 5 to 105 molecules/run (RoboGene M-bcr cDNA Quantification Module, Roboscreen). Values were normalized for expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the RoboGene GAPDH cDNA Quantification Module and c-ABL using the RoboGene c-Abl cDNA Quantification Module. Statistical analysis: Spearman's rank test was used to assess correlations between qPCR values using different controle genes. *Results.*Median values of qPCR and the availability of results at baseline, 3, 6 and 9 months are shown in Table 1.

Table 1.

	Baseline	*3 months*	*6 months*	*9 months*
qPCR (BCR-ABL/GAPDH) median range	0,6 0,006-0,8	2,5 0,001-22,4	0,52 0,002-4,8	0,21 0,0005-0,7
qPCR (BCR-ABL/cABL) median range	2,53 0,02-14,23	1,59 0,003-8,1	1,4 0,006-7,44	0,53 0,001-3,25

There was a highly significant correlation between BCR-ABL/GAPDH and BCR-ABL/cABL values (r=0,781, p<0,001, Spearman's rank test). In addition we also noticed a good correlations between number of BCR-ABL copies and BCR-ABL/GAPDH ratio (r=0,38, p<0,001) and between number of BCR-ABL copies and BCR-ABL/cABL ratio (r=0,49, p<0,001) as well. *Conclusions.* our data suggest that measurement of BCR-ABL/GAPDH and BCR-ABL/cABL ratios are the equivalent and usefull methods of molecular monitoring of CML treatment. BCR-ABL/GAPDH ratio significantly correlate with the value of BCR-ABL/cABL ratio. However, in spite of generally acceptable recommendations, we found also good correlations between the absolute number of BCR-ABL copies/run and BCR-ABL/GAPDH or cABL ratios.

0064

IMPROVED EFFICIENCY IN MOLECULAR DIAGNOSIS OF T(14;18)(Q32;Q21) AND T(11;14)(Q13;Q32) USING MULTIPLEX- AND LONG DISTANCE INVERSE-PCR

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Translocations t(14;18)(q32;q21) and t(11;14)(q13;q32) are cytogenetic hallmarks of follicular (FL) and mantle cell lymphoma (MCL), respectively. Both translocations target the joining region (JH) of the immunoglobulin heavy chain (IGH) locus at 14q32 resulting in juxtaposition of the BCL2 gene (18q21), or the BCL1 locus (11q13), to IGH. However, BCL2 and BCL1 breakpoints are only partially clustered so that a significant proportion of BCL2-IGH and BCL1-IGH fusion genes are not detected using standard PCR techniques. In order to identify and characterize the highest number of breakpoints, we used two sequential molecular approaches. First, a multiplex-PCR (M-PCR) assay was designed that allowed a single-tube amplification of the most common breakpoints in BCL2 (MBR and mcr regions) and BCL1 (MTC region), in addition to a control BCL2 fragment. Using this assay on 20 FL and 11 MCL diagnostic samples without knowledge of cytogenetic status, we observed 14 FL patients (70%) with BCL2-IGH (11 MBR and 3 mcr breakpoints) and 6 MCL patients (55%) with BCL1-IGH fusion genes. Sequencing of the corresponding PCR products confirmed the predicted fusion gene in every sample, revealing that M-PCR unequivocally identified the fusion gene in each patient. As a second approach in the remaining 11 negative patients, long distance inverse -PCR (LDI-PCR) was used to identify BCL2-IGH and BCL1-IGH rearrangements with variant breakpoint locations. PCR products corresponding to rearranged IGH alleles were cloned, sequenced, and the respective sequences compared to the GenBank database. We found a BCL2-IGH fusion in 3 patients with FL and a BCL1-IGH fusion in 3 patients with MCL (Table 1). The sequence of each fusion gene was confirmed by conventional PCR using a JH primer in combination with a BCL1 or BCL2 breakpoint region-specific primer. In BCL2, one of the breakpoints localized 1.5 kb upstream of the mcr region while the remaining localized at the intermediate cluster region and differed by 2 bp only. One of these breakpoints was identical to the one previously reported by others in 2 rare patients, suggesting that this particular sequence constitutes a micro cluster region of breakpoints. By con-

trast, BCL1 breakpoints were scattered within a 15 kb sequence at a distance of approximately 90 kb downstream of the MTC region. Two breakpoints localized within the promoter region of cyclin D1 at a distance of 1360 and 2870 bp from the transcription start site. In IGH, one translocation involved a D2-2/JH6 rearrangement, while the others showed involvement of either JH4 (4 cases) or JH6 (1 case). In summary, LDI-PCR was instrumental in the identification of an additional 3 breakpoints in BCL1 (60%) and 3 in BCL2 (50%) in patients who showed no breakpoints at the main cluster regions. Overall, BCL2-IGH fusion was detected in 17 patients with FL (85%) and BCL1-IGH fusion was found in 9 patients with MCL (82%). We conclude that M-PCR analysis for a fast determination of recurrent BCL1 and BCL2 breakpoints combined with LDI-PCR for assessment of variant breakpoint locations, is an effective approach for the diagnosis of t(11;14) and t(14;18) in non-Hodgkin lymphomas.

Table 1. BCL1- and BCL2-IGH sequences identified by LDI-PCR.

Acc. number	*Gene*	*Gene sequence*	*N region*	*IGH sequence*	*JH*
DQ401134	BCL1	GAGACTGGAAACTTTG	ctgtgaga gggc	ATATTGTAGTAGTACCAG CTGCTATACCACCTTTACT ACTACTACTACGGTATGG ACGTCTGGGGCCAAGGG	D2-TT 2/JH6
DQ400340	BCL1	TGTCATCAAACCACCG	cccctaat	TTTGACTACTGGGGCCAG	JH4
DQ241758	BCL1	CCTGGCGGCGACTTGA	ggtctc	GACTACTGGGGCCAGGG	JH4
DQ401133	BCL2	GTGAGAGTGCAGAATC	ggggagta	GGACGTCTGGGGCCAAG	JH6
DQ400339	BCLL2	ACAAGGCCTCTGCTAT	ggtt	CTACTGGGGCCAGGGA	JH4
DQ235270	BCL2	GAGAGTGCAGAATCTG	aagtctctg	ACTGGGGCCAGGGAACC	JH4

Molecular targeting and gene therapy

0065

THE EFFECT OF HISTONE DEACETYLASE INHIBITORS ON B-CELL DIFFERENTIATION IS NOT UNIQUE FOR TEL-AML1 POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIAS

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The TEL-AML1 fusion is the most frequently found translocation in childhood pediatric acute lymphoblastic leukemia (ALL). TEL-AML1-positivity is associated with L-asparaginase sensitivity and favorable clinical outcome in pediatric ALL. The fusion protein is thought to recruit co-repressors and histone deacetylases (HDACs), which in turn lead to transcriptional repression of AML1-responsive genes. FK228 (depsipeptide) is an HDAC inhibitor that affects the chromatine configuration and, as a consequence, affects gene transcription. We investigated whether HDAC inhibitors may be used to target TEL-AML1 positive ALL in children. To this aim, the *in vitro* cytotoxic effect of FK228 as single agent and in combination with L-asparaginase was tested in leukemic cells obtained from TEL-AML1 positive and negative children with ALL at initial diagnosis (by MTT-assay). In addition, the effect of FK228 on B-cell differentiation was analyzed by monitoring changes in differentiation marker expression using flow cytometry. Our data indicate that leukemic cells of 14 TEL-AML1 positive and 15 negative B-lineage ALL cases were both more *in vitro* sensitive to FK228 than normal bone marrow cells (p=0.03). FK228 exposure induced the differentiation of leukemic cells into more mature precursor B-cells. However, the *in vitro* cytotoxicity of FK228 did not differ between both ALL subtypes. FK228 had an additive but not a synergistic effect on *in vitro* sensitivity to L-asparaginase in both ALL subtypes. In conclusion, FK228 induces differentiation in children with B-lineage ALL, but its effect is not selective for TEL-AML1 rearranged B-lineage ALL only.

0066

IMATINIB MESYLATE CAN INDUCE MOLECULAR COMPLETE REMISSION IN IDIOPATHIC HYPEREOSINOPHILIC SYNDROME. A PHASE II MULTICENTRIC ITALIAN CLINICAL TRIAL

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Idiopathic hyper-eosinophilic syndrome (HES) is a rare haematological disorder characterized by persistent peripheral blood greater than 1,500 cells/mL lasting for more than 6 months, in the absence of other apparent aetiologies for eosinophilia with signs and symptoms of organ involvement. HES may be a reactive condition or a chronic myeloproliferative disorder with evidence of clonal proliferation, in which latter case it is usually referred to as chronic eosinophilic leukemia (CEL). Patients with HES generally have a poor prognosis, but the course of the disease may be variable. Severe visceral complications, including cardiopathies, are common and are often fatal illness. Treatment of HES includes corticosteroids, chemotherapeutic agents and, more recently, interferon-α (IFN-α). Cools *et al.* reported the involvement of PDGFRA, fused with FIP1L1, in a number of HES patients responsive to imatinib therapy. We treated with imatinib mesylate (100 to 400 mg daily) 59 patients affected by Hyper-Eosinophilic Syndrome (HES) enrolled in a multicentric Italian phase 2 clinical trial. All the patients were studied by molecular analysis for expression of FIP1L1-PDGFRA, TEL-PDGFRB, FGFR1-BCR and BCR-ABL chimerical transcripts. 23 patients (39%) were positive for the FIP1L1-PDGFRA rearrangement. Rapid, haematological complete responses (HCR) were recorded after one month of therapy in all FIP1L1-PDGFRA positive. In 36 patients resulted negative for FIP1L1-PDGFRA rearrangement we observed 8 (22%) hematological improvement (HI) and one HCR (HI+HCR 25%). Furthermore, a molecular complete remission (defined as the disappearance of FIP1L1-PDGFRA at qualitative RT-PCR evaluation) was recorded in all but one patients of the 23 valuable after three months of therapy, and we

obtained molecular remission in 18 out of 21 evaluable patients after six months of therapy. After one year of imatinib therapy, all 12 evaluable patients showed disappearance of the rearrangement. No significant toxicity was seen during the treatment. The median follow up was 7 months (range: 2-41). This is the largest series of HES patients treated with Imatinib with strong evidence of hematological and molecular effectiveness and absence of significant toxicity. This phase II study supports the use of Imatinib as first line therapy in FIP1L1-PDGFRA rearrangements positive HES patients. *Acknowledgments.* COFIN 2003 (Molecular therapy of Ph+ leukemias), by FIRB 2001, by the University of Bologna (60%), by the Italian Association for Cancer Research (A.I.R.C.), by the Italian National Research Council (C.N.R), by Fondazione Del Monte of Bologna and Ravenna (Italy) and A.I.L. grants, LeukemiaNet grants.

0067

SIRNA VERSUS LOCKED NUCLEIC ACID RNA ANTAGONISTS: STAY SINGLE!

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By the incorporation of Locked Nucleic Acid (LNA), a conformational analogue of RNA, single-stranded LNA/DNA oligonucleotides can be created which mimic RNA and have unrivalled gene silencing ability. Much discussion has centred on the utility and benefits of siRNA in both target validation and as a therapeutic option. This has been driven by significant publications including that of Soutcheck *et al.* (Nature 432, 173-177 2004), which demonstrated liver targeting as well as *in vivo* efficacy when siRNA against ApoB was tethered to a cholesterol moiety. We therefore sought to compare single-stranded oligonucleotide antagonists containing LNA with siRNA against this target in both *in vivo/in vitro* settings. The same motif used in the Soutcheck study was targeted with the LNA molecule, and the activity of unmodified siRNA was compared to the cholesterol-linked and native LNA molecules in their ability to down-regulate ApoB expression. LNA (SPC3197) inhibited ApoB expression by 90% while at an equimolar concentration siRNA was ineffective in the liver and jejunum. Cholesterol linked siRNA was partially effective in the jejunum (50% reduction in mRNA). Only the LNA mediated inhibition of ApoB expression was paralleled by decreases in serum cholesterol in the host animal. In a second model, siRNA molecules targeting Hif-1α mRNA (Yu *et al.* Lab Invest 84, 553-561 2004) were compared to a single-stranded LNA/DNA mixmer targeting Hif-1α, SPC2968. In *in vitro* analyses of these 2 molecules were equally potent. However, in a murine model the increased half-life of the LNA molecules translated to a potent inhibition of Hif-1α as measured by QPCR. This effect was observed in jejunum and liver, and persisted for at least 4 days. Hif-1α inhibition mediated by siRNA was not seen in any tissue analysed. Overall we demonstrate clear superiority of single chain LNA based RNA antagonists over siRNA for the use as therapeutic molecules.

0068

DUAL SRC/ABL INHIBITOR SKI-606 BINDING MODE IN BCR-ABL KINASE HYPOTHESIZED ON THE BASIS OF MOLECULAR DOCKING STUDIES

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Background. SKI-606 is a novel 4-anilino-3-quinolinecarbonitrile Src and Abl kinase inhibitor. SKI-606 has been shown to be a potent antiproliferative and proapoptotic agent when tested on Bcr-Abl-positive cell lines. The remarkable efficacy of SKI-606 against chronic myeloid leukemia (CML) cells in culture was mirrored by its activity *in vivo* against CML xenografts: K562 tumors regressed in nude mice when SKI-606 was administered per os once daily over a 5-day period. The crystal structure of the Bcr-Abl kinase domain in complex with SKI-606 has not yet been determined and the mode of binding of this inhibitor is therefore unknown. Moreover, there are currently no published data on the ability of SKI-606 to bind and efficiently inhibit the Bcr-Abl mutants known to confer resistance to imatinib. *Aims.* In this study, we used a molecular docking approach to a) determine SKI-606 binding mode to the wild-type (wt) form of the Bcr-Abl kinase; b) hypothesize SKI-606 binding mode to the more frequent, clinically relevant Bcr-Abl mutants known not to be inhibited by imatinib; c) predict which novel mutant forms might emerge and interfere with SKI-606 binding. *Methods.* Modelling of the human Abl kinase was performed with the program Modeller v7.7 (http://salilab.org/modeller) adopting the highly related Mus musculus Abl homologue as a template structure (PDB: 1OPJ, 0.175nm resolution). Chemsketch (http://www.acdlabs.com) was used to build a three-dimensional model of SKI-606. Flexible docking of the ligand to the protein was performed with Autodock v3.0 (http://www.scripps.edu/mb/olson). *Results.* We first docked SKI-606 on Bcr-Abl with the activation loop in the active (open) and inactive (closed) conformation (the latter is the one to which imatinib binds). According to our results, the interaction between SKI-606 and Bcr-Abl seems to be more stable when the activation loop is in the inactive conformation. The consequent structural study of SKI-606 modeled into wt-Bcr-Abl ATP binding site highlighted the variant residues located within a spherical environment of 0.5nm centered on SKI-606: Y253, T315 and F359 (residues numbered according to ABL exon Ia splice variant). The binding of SKI-606 to the eight Bcr-Abl mutants which are most frequently implicated in clinical resistance to imatinib mesylate was also studied: G250E, Y253H, E255K, T315I, M351T, F359V, H396R. Our results indicated that SKI-606 retains the ability of efficiently binding all the above mentioned Bcr-Abl variants with the exception of the T315I mutant. Finally, we identified six potential residues around SKI-606 that, if mutated, could potentially be able to interfere with the SKI-606/Bcr-Abl interaction: a) the charged residues K271, D381 and H361; b) the hydrophobic/aliphatic residues V299, A380 and M318. *Conclusions.* Pre-clinical data suggest that SKI-606 is a promising second-generation kinase inhibitor with potent antiproliferative and proapoptotic effects on CML cells. Our docking experiments indicate that SKI-606 may prove effective in imatinib-resistant patients since it is expected to retain the ability to bind several Bcr-Abl mutant forms. A phase I trial is about to start in CML and Philadelphia-positive acute lymphoblastic leukemia. Supported by European LeukemiaNet, COFIN 2003, FIRB 2001, AIRC, AIL, Fondazione del Monte di Bologna e Ravenna.

0069

EFFECTIVE INHIBITION OF BCR/ABL KINASE WITH TETRAMERIZATION DOMAIN DERIVED PEPTIDES MAPES TO COILED-COIL HELIX-ALPHA-2

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Background. As a result of the t(9;22), more than 95% of CMLs and 20-25% of adult ALLs express the p210(BCR-ABL) or the p185(BCR-ABL) fusion protein respectively. The BCR portion of the fusion protein harbors an N-terminal coiled-coil (CC) domain which induces tetramerization of BCR. The CC contains two α helical motifs - Helix-α-1 and Helix α-2 ' and assembles to dimers with antiparallel orientation which associate to form tetramers. Helix-α-2 contributes the majority of the dimer and tetramer interface. The BCR mediated tetramerization of ABL in the fusion protein leads to the constitutive activation of the ABL kinase. The subsequent permanent activation of multiple downstream signaling pathways induces the leukemic phenotype. Targeted inhibtion of BCR/ABL by the ABL kinase inhibitor Gleevec induces apoptosis in BCR-ABL transformed cells and leads to complete remission in CML and ALL patients. However, a large portion of ALL patients and CML patients in blast crisis relapse and acquire Gleevec resistant BCR/ABL mutations. It has been shown that Gleevec binds to the inactive conformation of the ABL-kinase which in case of the BCR/ABL fusion protein is present in monomers. We have previously shown that CC-derived peptides that interefere with BCR/ABL tetramer formation reduce the kinase activity and the transformation potential of BCR/ABL *in vivo*. Consequently the coexpression of CC-derived peptides results in an increased sensitivity of BCR/ABL expressing cells towards Gleevec by shifting the intracellular equilibrium towards BCR/ABL monomers. These CC-derived peptides provided a proove of concept that the tetramerization domain is a potential therapeutic target for BCR/ABL positive leukemia. *Aims.* Here we studied the inhibitory effects of the CC subdomain Helix-α-2 which harbors the majority of the protein-protein interface. We aimed to i) reduce the molecular weigth of the inhibitory peptides and ii) thereby to map the inhibitory effects of CC-derived peptides to CC-substructures. *Methods.* Helix-2-GFP fusion peptides were coexpressed with BCR/ABL in the IL-3 dependent cell line Ba/F3 using a bicistronic retroviral vector. The interaction of Helix-2 and BCR/ABL was checked by pull-down assays. The IL-3 independent proliferation of BCR/ABL-expressing Ba/F3 cells in presence of Helix-2 was studied in presence and absence of Gleevec. Anti-phospho-ABL specific immunoblotting was used to reveal the BCR/ABL autophosphorylation in these cells. All studies were performed with the previously published CC-GFP fusion

peptide as control. *Results.* Here we report that i) Helix-α-2 interacts with BCR/ABL to the same extend as the complete CC domain; ii) Helix-2 like CC decreases the autophosphorylation of BCR/ABL in transduced Ba/F3 cells; iii) Helix-2 increases the sensitivity of IL-3 independent BCR/ABL expressing Ba/F3 cells towards Gleevec to the same extend as CC; iv) Helix-2 shows no inhibitory effects on IL-3 independent Ba/F3 cells expressing activated c-Kit. *Conclusion.* Taken together these results show that Helix-α-2 specifically targets the tetramerization-interface of BCR/ABL. The peptides inhibit the ABL-kinase activity and enhance the inhibitory effects of Gleevec. This study provides important information for the use of Helix-α-2 as lead structure in the rationale design of small molecule inhibitors of BCR/ABL tetramerization.

0070

SINGLE-AGENT SU11657, A NOVEL FLT3 INHIBITOR, SHOWS BIOLOGIC ACTIVITY IN ACUTE MYELOID LEUKEMIA CELLS *IN VITRO*

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Background. fms-related tyrosine kinase3 (FLT3) is one of the most commonly mutated gene in human acute myeloid leukemia (AML) and has implicated in its pathogenesis. Constitutive activation of the FLT3 receptor tyrosine kinase, has been linked either by internal tandem duplication (ITD) of the juxtamembrane region or by point mutation in the second tyrosine kinase domain (TKD). *Aims.* The purpose of the study was to evaluate, *in vitro*, the effect and the biological activity of SU11657 (Pfizer), a new compound FLT3 kinase inhibitor. SU11657 was investigated on human cell lines from AML patients (MV4-11 and HL-60) and blast from patients AML using a wide range of concentrations (1nM-10 mM). *Methods.* FLT3 expression levels were evaluated by flow cytometry. Furthermore, to evaluate the effect of SU11657 we analyzed the cytotoxicity, induction of apoptosis and inhibition of cell proliferation by flow cytometry. The antiproliferative and cytostatic effects of SU11657 were confirmed by analysis of signal transduction. HL-60 cell line served as a control as it expresses a wild type receptor. MV4-11 is a cell line that expresses a naturally internal tandem duplication (-ITD) in homozygous form. *Results.* In HL-60 does not show relevant effect after treatment with SU11657. Instead, in MV4-11 we observed a decrease dose-dependent in cell viability after treatment with SU11657. The effects of this compound on cell cycle progression show an accumulation of G1/S phase and an induction of apoptosis at 1-10nM concentration after 24h of treatment. First we observed a dephosphorylation of FLT3 on Tyr(591) in whole cell extracts from MV4-11 cells after treatment with SU11657 100nM. We also demonstrated a hypophosphorylation of AKT on Ser(473) and a consequently dephosphorylation of BAD on Ser(136) at nanomolar concentration. We observed a dephosphorylation of STAT-5 to 100nM of SU11657 at 24h. We evaluated the effects of this new compound in AML primary progenitors that showed FLT3-ITD, FLT3-TKD and FLT3-wt. In the patients with mutation ITD and TKD was evident a modification of cell cycle progression with a decrease in G2/M phase and an increase of subdiploid peak. The effect of SU11657 in patients FLT3-wt was not relevant. *Conclusions.* Due to its FLT3 inhibitory activity, SU11657 represent promising compound for clinical studies in FLT3 mutation AML. Study of signal transductions and gene profile expression will contribute to further understanding of the drug mechanisms. *Acknowledgments.* COFIN 2005 (Myelodisplastic sindroms: pathogenetic models and promise of new therapies), COFIN 2003 (Molecular therapy of leukemias), by FIRB 2001, by the University of Bologna (60%), by the Italian Association for cancer research (A.I.R.C.), by the Italian National Research Council (C.N.R), by Fondazione Del Monte of Bologna e Ravenna (Italy) and A.I.L. grants.

0071

POTENTIAL THERAPEUTIC APPROACH OF RECOMBINANT TRAIL IN CML IN BLAST CRISIS

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Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL/APO2L) has been shown to induce apoptosis in a number of tumour cell lines as well as in some primary tumours whereas cells from most normal tissues are highly resistant to TRAIL-induced apoptosis. TRAIL is a member of the TNF super family and exerts its activity by interacting with a complex system of two death receptors (DR4/TRAIL-R1 and DR5/TRAIL-R2) and three decoy receptors (DcR1/TRAIL-R3, DcR2/TRAIL-R4 and osteoprotegerin/OPG). Although these receptors are characterized by a high sequence homology in their extracellular domains, only DR4/TRAIL-R1 and DR5/TRAIL-R2 contain a functionally active cytoplasmic death domain that allows an apoptotic response upon TRAIL stimulation. The biological significance of TRAIL as mediator of innate and specific immunity against transformed and virus-infected cells has been clearly documented by several reports. As membrane-bound TRAIL seems to have tumour-selective pro-apoptotic activity, the potential use of recombinant soluble forms of this molecule as cancer therapeutic is presently being exploited in several pre-clinical and preliminary clinical studies. Imatinib mesylate, a Bcr-Abl kinase inhibitor, has been very successful in the treatment of chronic myelogenous leukaemia (CML). However, the majority of patients achieving cytogenetic remissions with imatinib treatment have molecular evidence of persistent disease, and residual BCR/ABL$^+$ progenitors can be detected. There is a need to develop new approaches that enhance elimination of malignant progenitors in imatinib-treated patients. The aim of this work is to study the susceptibility of TRAIL-induced apoptosis in haematological neoplasias, namely in Chronic Myeloid Leukaemia (CML) in blast crisis For this purpose K562 cells were incubated in absence and presence of different concentrations of recombinant TRAIL as single agent or plus imatinib or MG262 (a proteasome inhibitor) during 72 hours. Cell death was evaluated by Annexin V/propidium iodide incorporation and detected by flow cytometry. The expression of TRAIL receptors and the proteins involved in apoptosis regulation, namely Bax, Bcl-2, p53 and survivin was analysed by flow cytometry using monoclonal antibodies. Preliminary results show that TRAIL as single agent doesn't decrease significantly K562 cell viability. However when the cells are previously treat with Imatinib or MG262 in lower concentration than IC50, we observe a potentiation of the cytotoxic effect. The increase in this cytotoxicity seems to occur by activation of apoptotic pathways as we have observed morphological characteristics of apoptosis and an increase in annexin V positive cells. The mechanisms involved may be related with the observed increase in Bax and/or in DR4 receptor expression. These results support the idea that imatinib and proteasome inhibitors may potentiate the apoptosis induced by TRAIL. On the other hand, the increase in imatinib efficacy, may allows its clinical use in lower doses with lesser toxicity. *Acknowledgments.* Profs. Catarina Oliveira,. Santos Rosa and Lina Carvalho Directors of Biochemistry, Immunology and Anatomopathology Institutes, respectively, Faculty of Medicine, University of Coimbra.

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0072

RCE1 DEFICIENCY ACCELERATES THE DEVELOPMENT OF A K-RASINDUCED MYELOPROLIFERATIVE DISEASE

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Background. Ras proteins undergo three processing steps that enhance membrane affinity: farnesylation, endoproteolysis, and carboxyl methylation of a carboxyl-terminal CAAX motif. Ras converting enzyme 1 (Rce1) catalyzes the second step the endoproteolytic removal of the three amino acids (AAX) downstream of the farnesylated cysteine. We have explored Rce1 as a potential drug target in the treatment of Ras-induced malignancies. We previously showed that inactivation of the Rce1 gene blocks the plasma membrane targeting of Ras and inhibits Ras transformation of fibroblasts and skin carcinoma cells *in vitro*. Moreover, the absence of Rce1 rendered Ras-transformed cells hypersensitive to a farnesyltransferase inhibitor. Currently, nothing is known about the impact of inhibiting Rce1 on the development of K-Ras-induced malignancies *in vivo*. *Aims.* Our aim was to test the hypothesis that inactivation of the Rce1 gene would inhibit the development, progression, and lethality of a K-Ras-induced myeloproliferative disease (MPD) *in vivo*. To accomplish this, we used Cre-loxP techniques in mice to simultaneously activate the expression of oncogenic K-Ras *to induce MPD* and inactivate the expression of Rce1. In this way, we could determine if the absence of Rce1 would block the development of K-Ras-induced MPD. *Methods.* We use mice that are heterozygous for an oncogenic mutation (G12D) in the Kras2 locus (Kras2LSL). In the absence of Cre recombinase, this mutant K-Ras allele is silent. Expression of Cre excises a 'floxed' transcriptional terminator sequence (LSL; loxP-STOP-loxP), turn-

ing on the expression of the oncogenic Kras2LSL allele. We and others have bred the Kras2LSL mice with mice harboring an interferon-inducible Mx1-Cre transgene and found that induction of Cre'by a single injection of an interferon-stimulant-activates the Kras2LSL allele in hematopoietic cells, causing a rapidly progressing, lethal MPD. We then bred Kras2LSLMx1-Cre mice on a background of homozygosity for a conditional Rce1 allele (i.e., Rce1flx/flxKras2LSLMx1-Cre). In those mice, induction of Cre simultaneously activated the Kras2LSL allele and inactivated Rce1. Controls were mice harboring a single conditional Rce1 allele (i.e., Rce1flx/+Kras2LSLMx1-Cre) in which Cre eliminated 50% of Rce1 expression. *Results.* As expected, expression of Cre in the control Rce1flx/+Kras2LSLMx1-Cre mice resulted in a rapidly progressing MPD with splenomegaly and overproduction of mature monocytes and granulocytes and an ability of hematopoietic cells to form colonies in methylcellulose in the absence of growth factors. In stark contrast, the inactivation of Rce1 in K-RasG12D-expressing cells accelerated the development of MPD, dramatically increased white blood cell counts and reduced survival. Moreover, Rce1-deficient K-RasG12D-expressing hematopoietic cells produced more and larger colonies in methylcellulose. The inactivation of Rce1 further resulted in a massive release of immature myeloid cells from the bone marrow which likely contributed to the early demise of the mice. *Conclusions.* Our hypothesis was that inhibition of Rce1 may be an effective strategy to block the development of K-Ras-induced malignancies. This hypothesis, which was based on comprehensive *in vitro* studies, was dashed by the current experiments. We find that inhibition of Rce1 *in vivo* actually accelerates the development of K-Ras-induced MPD. Future studies will evaluate the mechanism behind this totally unexpected result.

0073

OPTIMIZING T CELL RECEPTOR GENE TRANSFER TO VIRUS-SPECIFIC T CELLS FOR CLINICAL APPLICATION

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Patients with relapsed or resistant hematological malignancies after allogeneic stem cell transplantation (alloSCT) can be successfully treated by donor lymphocyte infusions (DLI). However, Graft-versus-Host Disease (GvHD) remains an important cause of morbidity and mortality. We previously showed that functional T cells with redirected anti-leukemic reactivity can be generated by transfer of T cell receptors (TCRs) specific for minor histocompatibility antigens (mHags) to virus-specific T cells. Adoptive transfer of virus-specific T cells to patients treated with alloSCT has a minimal risk for GvHD. The aim of this study is to develop a method for the generation of TCR-transduced virus-specific T cells for cellular immunotherapy of patients with relapsed hematological malignancies after alloSCT. Various single retroviral vectors were constructed containing the α and β chains of the HA-2 TCR linked by an IRES or 2A-like sequence. Introduction of a 2A-like sequence allows additional linkage of the human low affinity nerve growth factor receptor (NGFR) or CD20 selection marker genes by an IRES element. Inclusion of a selection marker gene allows purification of TCR-transduced cells, thereby reducing the risk for GvHD. The human CD20 gene also functions as suicide gene, allowing elimination of transduced cells *in vivo* when undesired side effects, like GvHD, occur. Since tetrameric complexes are currently not GMP-grade available, we explored the feasibility of using synthetic peptides for the generation of TCR-transduced virus-specific T cell lines. From various human individuals, $CD8^+$ cells were isolated and stimulated with a mixture of CMV and EBV peptides. At day 3, CD8+ cells were transduced with retroviral vectors encoding the HA-2 TCR. Due to selective expansion upon peptide stimulation, all cell lines were shown to contain high cumulative percentages of virus-specific T cells (20-80%) as well as TCR-transduced cells (10-50%) at day 8-12. Moreover, significant numbers of specific T cells were obtained, demonstrating that this strategy is feasible for adoptive cellular immunotherapy. Highest levels of TCR expression and HA-2-specific lysis were obtained with retroviral vectors containing two genes encoding the TCR alpha and β chains linked by an IRES or 2A-like sequence. Upon linkage of a third gene, TCR expression and HA-2-specific lysis were slightly (NGFR) or significantly (CD20) reduced. Since highly-enriched virus-specific T cells will be used for adoptive transfer, GvHD is not likely to develop and inclusion of a selection marker/suicide gene not strictly required. Therefore, for clinical application of TCR-transduced virus-specific T cells, a retroviral vector containing two genes encoding the TCR α and β chains in the absence of a selection marker/suicide gene is preferable.

0074

DUAL SPECIFIC T CELLS CHANGE THEIR T CELL RECEPTOR (TCR) CELL SURFACE DISTRIBUTION UPON TCR TRIGGERING

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TCR transfer to engineer tumor specific T cells may be an alternative strategy for adoptive immunotherapy. When virus specific T cells are used for TCR transfer, we hypothesize that due to the latent presence of viral antigens the survival of TCR-transferred dual specific T cells will improve. However, repetitive stimulation of the endogenous TCR may lead to selection of dual specific T cells with high expression of the endogenous TCR and low expression of the introduced TCR. To address this issue, we used CMV-specific T cells that were transduced with the hematopoietic minor histocompatibility antigen HA-2 specific TCR. The dual specific T cells were repetitively stimulated either via their endogenous virus specific TCR or via the introduced HA-2 specific TCR and analysed by FACS after each stimulation. In time, the expression of the endogenous and introduced TCRs measured with CMV and HA-2 tetrameric complexes diverged. Repetitive stimulation of the endogenous TCR skewed the dual specific T cells towards a cell population that primarily expressed the endogenous TCR. In contrast, repetitive stimulation of the introduced TCR skewed the T cells towards T cells that primarily expressed the introduced TCR. However, this divergence in tetramer stainings appeared to revert quickly after stimulation via the other TCR, suggesting that this divergence was the result of a difference in TCR cell surface distribution and not of selective outgrowth of different T cells. To rule out that differences in tetramer stainings were the result of selective outgrowth, T cells were sorted after repetitive stimulation expressing primarily the endogenous or introduced TCR. These cells were subsequently stimulated on the endogenous or introduced TCR and analysed for TCR expression and functional activity. Results indicate that no selective outgrowth occurred, but that T cells change their TCR cell surface distribution dependent on which TCR is triggered. In conclusion, virus specific TCR-transferred T cells repetitively stimulated via their endogenous TCR phenotypically seemed to express primarily the virus specific TCR. However, when restimulated on the introduced TCR, T cells reverted into cells with high expression of the introduced TCR that exerted potent HA-2 specific anti-leukemic activity, indicating that these dual specific T cells are useful for clinical applications.

0075

RELEVANCE OF MEK/ERK ACTIVATION IN THE MAINTENANCE OF THE PML/RAR-ALPHA INTEGRITY IN ACUTE PROMYELOCYTIC LEUKEMIA

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The mitogen-activated protein kinase MEK/ERK and phosphatidylinositol 3-kinase PI3K/Akt pathways are involved in proliferation, inhibition of apoptosis, differentiation and cell survival. These pathways are frequently activated in Acute Promyelocytic Leukemia (APL), and play a key role in the survival of neoplasic cells. The hallmark of APL is the t (15; 17), which leads to the expression of the PML/RARα fusion protein. PML/RARα is the central leukemia-inducing lesion in APL and is directly targeted by all-trans retinoic acid (ATRA) as well as by arsenic trioxide (ATO), both compounds able to induce clinical complete remissions. Nevertheless, the precise intracellular mechanisms of action of ATRA and ATO remain unclear. The purpose of this study was to evaluate: 1) the effects induced by the downmodulation of MEK/ERK and PI3K/Akt pathways on PML/RARα expression, and 2) the role of these pathways in the PML/RARα degradation induced by ATRA and low-dose ATO in APL cells. PML/RARα expression was analyzed by western blot after treatment of the promyelocytic cell line NB4 with the selective pharmacological inhibitors of MEK/ERK and PI3K/Akt pathways, PD98059 (20 μM) and LY294002 (20 uM) respectively, given either alone or in combination with ATRA (1uM) or ATO (0.1 μM). The inhibitor of the MEK/ERK pathway caused a significant degradation of PML/RARα, which was reversed after treatment with the general caspase inhibitor z-VAD-fmk (50 μM), indicating that PML/RARα degradation induced by downmodulation of MEK/ERK seems to be a mechanism dependent on caspase activation. In addition, the combined treatment with ATRA or

ATO and PD98059 further reinforced the PML/RARα degradation induced by ATRA or low doses of ATO. On the other hand, the combined treatment with arsenic trioxide and LY294002 reversed oncoprotein degradation induced by ATO alone, thus suggesting that the PI3K/Akt pathway might mediate the degradation of PML/RARα induced by low-dose ATO. Taken together our findings suggest that MEK/ERK activation might be responsible for the maintenance of the PML/RARα integrity in APL cells. The results reported encourage the use of anti MEK/ERK reagents in clinical trials for the treatment of APL, either as single agents or in association with retinoids or arsenic compounds.

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0076

TRANSCRIPTIONAL PROFILING OF PROTEASOME INHIBITOR-MEDIATED ANTI-TUMOR ACTIVITY IN EPSTEIN-BARR VIRUS-INFECTED NASAL NK LYMPHOMA CELLS

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Background. Natural killer (NK) cell lymphoma is frequently associated with Epstein-Barr virus (EBV), and activated NF-kappa B is a critical mechanism by which EBV-infected lymphoma cells are protected from apoptotic stress. The proteasome inhibitor, bortezomib, abrogates degradation of IkB, which blocks the transcriptional activity of NK-kB. This effect may account in part for anti-tumor effects in several types of cancer. *Aim.* To determine the precise link between inhibition of proteasomal degradation and induction of apoptosis on EBV-related NK cell malignancy. *Method.* By using DNA microarray (Affymetrix U133plus2.0 chip), we investigated the molecular pathway which may be linked to the anti-tumor effects of bortezomib on NK cell lymphoma cell line with EBV latency type II infection (designated as SNK-6). *Results.* Transcriptional profile of bortezomib-treated SNK-6 cells involved down-regulation of growth/survival signaling, up-regulation of molecules related to induction of apoptosis, as well as up-regulation of heat shock proteins and the ubiquitin/proteasome pathway, such as ubiquitin-specific peptidase 7 (USP7; herpes virus-associated). *Conclusion.* Our results demonstrated that proteasome inhibition elicits activation of multiple signaling pathways, and provides novel insight into the bortezomib mediated anti-tumor activity in EBV-associated NK lymphoma cells.

0077

SIRNA-MEDIATED MLL-AF4 KNOCKDOWN AFFECTS TERT EXPRESSION

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Background. The reciprocal chromosomal translocation t(4;11)(q21;q23) results in the expression of two fusion-proteins, MLL-AF4 and AF4-MLL, and marks a therapy-resistant infant acute lymphoblastic leukaemia, where new ways of treatment must be sought. *Aim.* We addressed the effects of siRNA-mediated MLL-AF4 suppression on the expression of putative target genes in t(4;11)-positve leukaemic cell lines. *Methods.* t(4;11)-positive cell lines were electroporated with MLL-AF4, HOXA7 or control siRNAs. Gene expression was analyzed by real time RT PCR, intracellular protein-DNA interactions by chromatin immunoprecipitation, and promoter methylation status by bisulfite sequencing. *Results.* SiRNA-mediated suppression of both MLL-AF4 and of HOXA7, a putative target gene of MLL-AF4, diminishes hTERT transcript levels. In particular, the knock-down of MLL-AF4 is associated by changes in the methylation status of the hTERT promoter. Furthermore, chromatin immunoprecipitation (ChIP) provides evidence for direct binding of HOXA7 to the hTERT promoter. Current experiments address a possible direct effect of MLL-AF4 on hTERT expression. Furthermore, we are studying the role of HOXA7 and hTERT in MLL-AF4-mediated repression of apoptosis. *Conclusions.* Our results suggest that MLL-AF4 controls hTERT expression at least in part via HOXA7. The observed changes in methylation pattern of the hTERT promoter upon MLL-AF4 depletion supports a function of this leukaemic fusion protein in the epigenetic control of gene expression. Analysis of intracellular pathways may not only elucidate the oncogenic action of MLL/AF4 but may also open the avenue for new treatment options by targeting MLL-AF4 key functions in infant leukaemia.

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0078

PRENYLATION INHIBITORS MODULATE IL-6 AND IGF-1 DEPENDENT SIGNALING IN MULTIPLE MYELOMA CELLS

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Background. Multiple myeloma (MM) is a fatal hematologic malignancy associated with disruption of RAS-to MAP kinase (MAPK/ERK) signaling. IL-6 and IGF-1 promote malignant plasma cell proliferation through stimulation of MAPK and PI-3 kinase/AKT signaling. Prenylation inhibitors such as farnesyltransferase inhibitors (FTIs), geranylgeranyl transferase inhibitors (GGTIs) and lovastatin block RAS post-translational modification and disrupt RAS signaling. *Aims.* To assess the efficacy of prenylation inhibitors (e.g. FTI L-744,832, GGTI-2147 and lovastatin) in blocking MM cell responses to IL-6 and IGF-1. *Methods.* Primary MM cells were isolated by magnetic cell sorting using CD138-coupled microbeads. MM cells were titrated with prenylation inhibitors and IC50´s were determined by proliferation assays (MTS). Synergistic inhibition of MM cell proliferation upon co-treating with FTI and GGTI or lovastatin was evaluated using the CalcuSyn program. Levels of IL-6- and IGF-1-induced phosphorylated MEK-1/2 and MAPK-1/2 were detected by Western blotting. *Results.* FTI L-744,832 inhibited growth of MM cell line NCI-H929 cultured with IL-6 or IGF-1 even more potently than in cytokine/growth factor-free medium (IC50s 1.3 μM, 1.8 μM and 4.2 μM, respectively). IL-6 moderately protected NCI-H929 cells from inhibitory effects of GGTI-2147, while IGF-1 had no effect (IC50s 1.1 μM and 0.5 μM vs. 0.5 μM, respectively). IL-6 and IGF-1 protected NCI-H929 cells from lovastatin-induced growth inhibition (IC50s 4.7 μM and 5.0 μM vs. 1.4 μM, respectively). Co-treating NCI-H929 cells with FTI L-744,832 and GGTI-2147 or lovastatin synergistically inhibited proliferation regardless of the presence of IL-6 or IGF-1. In primary MM cells (n=7), FTI L-744,832 elicited anti-myeloma effects only at concentrations much higher than those found to inhibit healthy donor CD34+ cells (IC50´s 51-396 μM vs. 8.2μM) and thus may be ineffective or cause non-specific toxicity when used as a single agent. However, GGTI-2147 and lovastatin induced specific anti-myeloma activity in some cases. Furthermore, combination of FTI with GGTI or lovastatin synergistically inhibited primary MM cell proliferation (IC50´s 0.6-23.1 μM). Activating RAS mutations (4 K-RAS, 1 N-RAS; one sample had both K- and N-RAS mutations) were found in 4/7 (57%) MM patient samples, but anti-myeloma activity of prenylation inhibitors could not be correlated to RAS mutation status. Western blotting demonstrated that FTI/GGTI or FTI/lovastatin co-treatment more completely blocked activation of MEK-1/2 and MAPK-1/2 in NCI-H929 cells than treatment with any of the compounds alone. Furthermore, co-treatment elicited greater inhibition of IL-6 and IGF-1 induced MEK-1/2 and MAPK-1/2 activation in NCI-H929 cells. IL-6, IGF-1 and prenylation inhibitors did not affect AKT phosphorylation status in NCI-H929 cells. *Summary/Conclusions*. Our results support that inhibition of RAS down-stream signaling is a major mechanism through which FTI/GGTI and FTI/lovastatin co-treatment synergistically inhibit MM cell proliferation, even in the presence of cytokines and growth factors known to promote MM cell growth (e.g. IL-6 and IGF-1). Alternative prenylation of K- and N-RAS by GGTase I in the presence of FTIs may explain the clinically observed incomplete response to FTI treatment. As the majority of RAS mutations in multiple myeloma occur in K- and N-RAS, FTI-resistance due to alternative geranylgeranylation may have therapeutic consequences in this disease.

0079

EFFECTS OF BORTEZOMIB IN APOPTOSIS IN B-CLL CELLS INDEPENDENTLY OF PRIOR THERAPY - CHARACTERIZATION OF BIOCHEMICAL MECHANISMS ASSOCIATED WITH THE RESPONSE

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B-cell lymphocytic leukaemia (B-CLL) is the most common adult leukaemia in the Western world. Although nucleoside analogues such as fludarabine and 2-chlorodeoxyadenosine (cladribine) have excellent activity in patients who have not received prior therapy, their impact on long-term survival is unclear and few agents display any activity in refractory disease. Nonetheless, the available evidence suggests that CLL emerges primarily as the result of deregulated apoptosis rather than unchecked

proliferation. Thus, agents that selectively target the survival pathway(s) active in B-CLL cells could reverse drug resistance. Proteasome plays a pivotal role in the control of many apoptotic and cell cycle-regulatory processes, become the focus of new approaches to the treatment of cancer, including B-cell malignancies. Bortezomib is the first proteasome inhibitor approved by FDA and EMEA for the treatment of refractory multiple myeloma. Extensive preclinical data is being developed to study the potential therapeutic of this new drug in other cancers. Our previous results show that BZ induces apoptosis as single agent, in a dose dependent manner, showing some selectivity for the transformed cells (EHA, 2005). Our preliminary results support the idea that the Bortezomib apoptotic effect may occur in a Bax dependent way (ASH, 2005). Here we examined the effects of bortezomib on apoptosis in peripheral blood mononuclear cell isolates from patients with CLL and characterized some of the biochemical mechanisms associated with the response. Mononuclear cells isolated from the blood of 24 CLL patients (14 without and 10 with prior conventional therapy, chlorambucil or fludarabine, 2 of those with refractory disease) were treated *in vitro* with bortezomib (ranging concentration from 0.1 nM to 10 μM), and evaluated for apoptosis by flow cytometry. Directly conjugated monoclonal antibodies to CD5 and CD19 were used to identify LLC-B cells. The expression of some proteins involved in mitochondria and membrane apoptotic pathways, namely the Bcl-2 proteins family, Bax and Bcl-2, the suppressor protein p53, the IAP survivin and TRAIL receptors was determined by flow cytometry using monoclonal antibodies. At 24 h incubation time, bortezomib induces apoptosis in isolated cells from patients without and with prior conventional therapy. However an average increase in the percentage of apoptotic cells versus patients with prior therapy were observed (±25%) witch may be related with a higher Bax expression. Our preliminary results also observed an increase in survivin expression in Bortezomib treated cells witch, if confirmed, supports the idea that Bortezomib induces apoptosis by an independent membrane apoptotic pathway. On the other hand, the apoptotic effect seems to be independent of basal TRAIL receptors expression. Our data confirm that bortezomib, like other proteasome inhibitors, has proapoptotic activity in CLL cells. More importantly, bortezomib was also effective in B-CLL cells isolated from refractory patients. Although the biochemical mechanisms and the extent of this activity and whether or not it translates into clinical benefit will only be known after patients enrolled on a trial have been evaluate.

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0080

NO EVIDENCE FOR CONSTITUTIVELY ACTIVATED FLT3 IN JUVENILE MYELO-MONOCYTIC LEUKEMIA

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Background. Activating FLT3 mutations have been identified as prognostic factors in several myeloid malignancies. Recent studies have demonstrated that ligand-independent activation of FLT3 can also result from overexpression of wild-type FLT3. In addition, ligand-dependent activation has been observed in leukemic cells co-expressing FLT3 ligand (FLT3L), resulting in autocrine FLT3 signaling which is independent of FLT3 mutations. *Aims.* In Juvenile Myelo-Monocytic Leukemia (JMML), FLT3 internal tandem duplications (FLT3/ITDs) and mutations affecting the tyrosine kinase domain (TKD) are rare. However, no data are yet avialable on the frequency of expression of FLT3 and FLT3L in JMML. If activated FLT3 occurs in JMML these patients might benefit from treatment with small molecule FLT3 inhibitors, especially as the curative treatment of JMML is limited to allogeneous stem cell transplantation. *Methods.* The presence of activating FLT3/ITDs and FLT3/TKD mutations were screened in 51 JMML patients. In 21 patients FLT3 and FLT3L mRNA expression were assessed by real-time quantitative PCR (Taqman). MTT assays were performed to assess the sensitivity of JMML cells to the FLT3 inhibitor PKC412. *Results.* In none of the 51 JMML samples FLT3-ITDs or TKD mutations were found. FLT3 appeared to be expressed only at basal levels and FLT3L expression was very low. Consistent with the absence of mutatons and lack of FLT3 and FLT3L expression, no PKC 412 cytotoxicity was found in the JMML samples (n=12), in contrast to leukemic cells of infants with MLL-rearranged ALL which expressed activated FLT3. *Conclusions.* These data suggest that constitutively activated FLT3 does not occur in JMML. Therefore targeting FLT3 by tyrosine kinase inhibitors like PKC412 is unlikely to be effective in JMML.

0081

MOLECULAR TARGETS OF THE PROTEASOME INHIBITOR, BORTEZOMIB, ON ADULT T-CELL LEUKEMIA CELLS

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Background. The ubiquitin-proteasome system (UPS) is critical for regulation of fundamental cellular systems, such as cell cycle regulation, death, and immune response. The molecular mechanism of proteasome inhibitor-mediated anti-cancer activity has recently been extensively studied, and one of the major pathways is inhibition of the NF-kB cascade. Adult T-cell leukemia (ATL) is a fatal neoplasia derived from HTLV-1 infected T lymphocytes, and NF-κB activation is frequently associated with HTLV-1 infection. *Aim.*We therefore sought to determine the antitumor effect of the proteasome inhibitor, bortezomib in ATL cells, using gene expression profiling. METHODS and *Results.* Assessment of gene regulation by microarray analysis revealed that down-regulation of genes involved in anti-apoptosis (i.e., BCL2, and IAP5), up-regulation of genes related with apoptosis (i.e., FAF1 and TNFRSF10B), heat shock proteins (i.e., HSPA, HSPCA), and oxygen stress (i.e., heme oxygenase-1). Since heme oxygenase-1 is believed to represent a key enzyme for the protection of cells against *stress*, it provides a growth advantage and contributes to cellular resistance against chemotherapy. *Conclusion.* Our results suggest that specific inhibition of heme oxygenase-1 expression in combination with proteasome inhibitor may be a new option in treating ATL patients and may be used as a sensitizer for chemotherapy.

0082

ANTISENSE THERAPY AGAINST MULTIDRUG RESISTANT GENE IN ACUTE MYELOBLASTIC LEUKEMIA CELL LINE

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Background. Acute myeloblastic leukemia (AML) is the most common leukemia in adults. Although the clinical outcome of acute leukemia has been proved by recent progress in chemotherapy, it is still a difficult disease to treat. One major problem is the emergence of leukemic blast cells that are resistant to anticancer drugs. This phenomenon is named multidrug resistance. A representative cause of MDR is the expression of the MDR1 gene and its product ,P-glycoprotein (Pgp) on the cell surface membrane. Expression of Pgp is associated with its resistance to several types of antineoplastic agents such as anthracyclines, taxans, epipodophylotoxines and vinca alkaloids. *Aim.*In this study we tried to reverse MDR phenotype in leukemic cells by antisense in complex to nanoparticle (PEI) against MDR1 gene. *Methods.* In the present study, the Pgp expressing cell line was established from parental K562 cell line with increasing concentrations of Doxorubicin starting with 5 ng/mL. The Pgp expressing cell line was obtained in 20 ng/mL and named KDI/20. In order to reverse the MDR phenotype due to Pgpexpression four different sequences of sense, antisense and one random sequence with phosphorothioate (PTO) modification (PS-ODN) against MDR1/mRNA was synthesized. They were treated on the KDI/20 in combination with two nonviral vectors: 1) Fugene 6 transfection reagent (cationic lipid0 and 2) polyethylenimine (a cationic polymer, nanoparticle). The effect of PS-ODN was assessed at the cellular level by flowcytometry for Pgp detection, rhodamin 123 assay for functional assessment of Pgp, RT-PCR at the molecular level for MDR1/mRNA and MTT assay in order to assess the sensitivity of cells to Doxorubicin. *Results.* the results showed a decrease in the percentage of Pgp protein and MDR1/mRNA expression and an increase in the accumulation of Rh 123 and drug sensitivity of cells to Doxorubicin by antisense I and III. Summary: The results showed that antisense can reverse MDR phenotype at transcription level and the PEI vector is more efficient than cationic lipid.

0083

IDENTIFICATION OF HSP-32 = HEME OXYGENASE-1 AS A NOVEL KIT D816V-DEPENDENT TARGET IN NEOPLASTIC MAST CELLS IN SYSTEMIC MASTOCYTOSIS

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Background and Aims. Systemic mastoyctosis is a myeloid neoplasm characterized by abnormal growth and accumulation of mast cells (MC) in visceral organs. In most patients, the D816V-mutated variant of KIT, which mediates resistance against most available tyrosine kinase inhibitors, is found. Therefore current research is focusing on novel targets in MC. We analyzed expression and function of the survival factor Hsp-32 (= heme oxygenase-1, HO-1) in neoplastic MC. Methods & *Results.* As assessed by Northern blotting and RT-PCR, the human MC line HMC-1 that exhibits KIT D816V, was found to express Hsp-32 mRNA. Expression of the Hsp-32 protein in neoplastic MC was demonstrable by immunocytochemistry and Western blotting. The Hsp-32 inductor hemin (10 μM) increased the expression of Hsp-32 in HMC-1 cells. To examine the role of mutated KIT in expression of Hsp-32, Ba/F3 cells with doxycycline-inducible expression of KIT D816V were employed. In these experiments, KIT D816V was found to induce Hsp-32 promoter activity as well as the expression of Hsp-32 mRNA and the Hsp-32 protein in these cells. The KIT D816V-induced upregulation of Hsp-32 in Ba/F3 cells was completely blocked by a combination of LY294002 (PI3-kinase inhibitor) and PD89059 (MEK inhibitor), but not by single signal transduction inhibitors, suggesting involvement of multiple signaling pathways in KIT D816V-induced Hsp-32 expression. We next examined whether targeting of Hsp-32 is associated with decreased survival. As assessed by 3H-thymidine uptake, the Hsp-32 inhibitor pegylated zinc-protoporphyrin (PEG-ZnPP) reduced the proliferation of HMC-1 cells in a dose-dependent manner (IC50: 5 μM). The PEG-ZnPP-induced inhibition of growth was found to be associated with induction of apoptosis in HMC-1 cells (control: 1±0.6 vs PEG-ZnPP, 5 μM: 55±5% apoptotic cells, $p<0.05$). *Conclusions.* Our data show that Hsp-32 is a novel survival factor and interesting target in neoplastic human MC exhibiting the D816V-mutated variant of KIT.

Chronic myeloproliferative disorders I

0084

EPHA3 TYROSINE KINASE RECEPTORS AS TARGETS IN CHRONIC MYELOPROLIFERATIVE DISEASES

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Background. Eph receptors tyrosine kinase and their ephrin ligands, highly expressed during embryogenesis are involved in many key developmental processes. Eph/ephrin interaction triggers a bidirectional signals transduction cascade that regulates morphogenesis and cell-cell interaction. Although Ephs receptors are not detectable in normal adult tissues, they are overexpressed in many tumors, suggesting a possible role of these PTKs in oncogenesis. Activation of tyrosine kinases and cell-signal transduction pathways are of increasing interest in the pathogenesis of chronic myeloproliferative disorders (CMPD). Dasatinib (BMS-354825), a novel BCR-ABL inhibitor exhibits an interesting inhibitory activity on some tyrosine kinases. *Aim.*The aim of this study was to comprehensively evaluate the expression of EphA3 in CMPD and investigate the possibility of exploiting EphA3 as a therapeutic target of BMS-354825. *Methods.* EphA3 mRNA expression was analyzed, using Real Time PCR, in 266 samples obtained from CMPD patients (133 PB and 133 BM), 43 with a diagnosis of PV, 35 ET, 20 IM, 24 CMML, 4 HES, 50 CML in chronic phase and 90 patients with a diagnosis of Ph-CMPD. 38 normal controls (18 PB and 20 BM) were also evaluated. Moreover, we investigated the expression level of EphA3 in 35 sample of B-CLL, 39 AML, 27 ALL and in 7 cell lines (Jurkat, K562, HL-60, MEL, NIH-3T3, 293T, COS-7). Protein expression and localization were examined using Western Blot, Immunoprecipitation and Immunofluorescence analysis with appropriate antibodies. Transient transfection was performed in 293T e COS EphA3- cells using EphA3 plasmid. Nucleotide sequencing of tyrosine kinase catalytic domain was performed in 45 EphA3+ patients and in Jurkat cells. BMS incubation of normal/pathological samples and cell lines was performed (3,10,20 nM). Cells proliferation was evaluated using MTT assay; apoptosis rate was analyzed by FACS (Annexin V) and colony growth was examined on methylcellulose culture. *Results.* We found EphA3 overexpression in Ph- mieloproliferative patients (45%) compared to normal controls (5%) [p=0,004 in the PB e p=0,005 in the BM], with a significantly difference in the amount of transcript. 14% of B-CLL, 40% of ALL, 30% of AML and 8% of CML were positive. The overexpression was observed more frequently in BM as compared to PB (51,5% vs 22,8%). No expression difference was noted among the Ph-CMPD. Western Blot analysis confirmed protein expression in EphA3+ samples and revealed receptor phophorylation. Dasatinib led to significant dose-dependent inhibition of EphA3 phosphorylation. Moreover, BMS induced significant apoptosis (mean value 32%), colony growth reduction (mean value of 34,2 vs 76,5) and proliferation rate inhibition (48%) of EphA3+ cells compared to normal controls. Immunofluoresce assay showed transmembrane localization of EphA3 receptor and revealed cells projections reduction, cell repulsion and cell rounding only in EphA3+ transfected cells. No kinase domain mutations were found in EphA3 overexpressing patients and Jurkat cells studied. Conclusion: EphA3 is abnormally expressed in different hematological malignancies with a significant overexpression in CMPD as compared to normal controls. EphA3 phosphorylation blocking induced by BMS-354825 results in growth arrest and apoptosis of EphA3 overexpressing cells. Therefore, EphA3 may represent a potential candidate for targeted signal transduction therapy.

0085

IDENTIFICATION OF MCL-1 AS A NOVEL TARGET IN NEOPLASTIC HUMAN MAST CELLS: EVIDENCE FOR COOPERATIVE GROWTH-INHIBITORY EFFECTS OF MCL-1 ANTISENSE OLIGONUCLEOTIDES, PKC412, AND AMN107

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Background. Mcl-1 is a Bcl-2 family-member that has been described to act anti-apoptotic in various myeloid neoplasms and therefore has been

proposed as a potential therapeutic target. Systemic mastocytosis (SM) is a myeloid neoplasm involving myelomastocytic progenitors. *Aims.* We examined the expression and functional role of Mcl-1 in neoplastic mast cells (MC), to determine whether Mcl-1 could serve as a target in MC neoplasms. *Methods.* As assessed by RT-PCR and immunohistochemistry, primary neoplastic MC were found to express Mcl-1 mRNA and the Mcl-1 protein in a constitutive manner in all patients analyzed. Moreover, the human MC-leukemia cell line HMC-1 was found to express Mcl-1. Transfection of these cells with Mcl-1-specific antisense oligonucleotides (ASO) or an mcl-1-specific siRNA using lipofectin resulted in a reduced survival and increased percentage of apoptotic cells compared to control. *Results.* The effects of mcl-1 ASO were seen with the HMC-1.1 subclone carrying the G560V c-kit mutation (mcl-1 ASO, 250 nM: 49±4% apoptotic cells compared to control: 3±2%, $p<0.05$; mcl-1 siRNA: 41±5% vs control: 5±3%, $p<0.05$) as well as with HMC-1.2 cells carrying both the G560V c-kit mutation and the D816V c-kit mutation (mcl-1 ASO, 250 nM: 36±2% apoptotic cells compared to control: 6±1%, $p<0.05$; mcl-1 siRNA: 30±6% vs control: 5±2%, $p<0.05$). Moreover, mcl-1 ASO were found to cooperate with the tyrosine kinase inhibitors (Novartis Pharma AG) imatinib, AMN107, and PKC412 in producing growth inhibition in HMC-1.2 cells. *Summary*. Together, these data show that Mcl-1 is a novel survival factor and attractive target in neoplastic human MC. Whether the Mcl-1-targeting concept can be developed far enough to reach clinical application remains to be elucidated.

0086

ANAGRELIDE: STUDIES ON ITS MODE OF ACTION USING DIFFERENT MODEL SYSTEMS OF MEGAKARYOCYTE DIFFERENTIATION

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Background and Aims. Anagrelide is a potent and selective inhibitor of megakaryocytopoiesis used for the treatment of essential thrombocythaemia. Although the effectiveness of this drug in lowering platelet counts is now firmly established, its primary mechanism of action remains elusive. We have previously demonstrated that anagrelide inhibits the development of megakaryocytes from isolated CD34 positive haematopoietic progenitors (Wang *et al.* Br. J. Pharmacol. 2005;146:324). Given that the use of cell lines could facilitate the identification of the molecular target of anagrelide, in this study we have compared the effects of the drug on the proliferation and differentiation of CD34 positive cells with those observed in UT7/mpl, a growth factor-dependent haematopoietic cell line engineered to express the human thrombopoietin (TPO) receptor MPL. *Methods.* CD34 positive cells were purified from human umbilical cord blood and cultured for 12 days in IMDM-based medium supplemented with 20 ng/mL TPO. UT7/mpl cells were maintained in exponential growth in α-MEM containing G418 and 2 ng/mL GM-CSF. To induce megakaryocytic differentiation in UT7/mpl cells GM-CSF was replaced by 20-100 ng/mL TPO; alternatively cells were treated in the presence of GM-CSF with 10 nM phorbol-myristyl-acetate (PMA). *Results.* Culture of UT7/mpl for 5 days with GM-CSF or TPO led to >10-fold cell expansion. Addition of anagrelide at 1 μM, a concentration which causes maximal inhibition of megakaryocytopoiesis in CD34 positive cell cultures and corresponds to >10-fold its IC(50) in that system, caused only a slight and non-consistent inhibition of UT7/mpl cell expansion (10-26% in cells grown with GM-CSF and -8 to +17% in cells grown with TPO). In addition, flow cytometric analysis showed that, unlike its effect in CD34 positive cell cultures, in UT7/mpl cells anagrelide did not inhibit TPO-induced expression of the megakaryocytic differentiation marker CD61. Furthermore, the lack of anagrelide activity was unrelated to the concentration of TPO used. Since UT7/mpl cells undergo megakaryocytic differentiation also when treated with phorbol esters, the activity of anagrelide against this class of agents was also tested. PMA completely inhibited UT7/mpl cell growth, caused a marked enlargement in cell size and induced a >3-fold increase in the expression of CD61. Addition of 1 μM anagrelide had no significant effect on any of these parameters. *Conclusions.* These findings indicate that UT7/mpl cells cannot replace normal haematopoietic progenitors as an *in vitro* model system to study the mechanism by which therapeutic doses of anagrelide inhibit megakaryocyte differentiation. In addition, our study suggests that the molecular target of anagrelide lies further down stream from the ligand binding site of MPL. Since PMA induces megakaryocytic differentiation through activation of protein kinase C, our study further suggests that this pathway is not a target of anagrelide.

0087

TISSUE-PLASMINOGEN ACTIVATOR AND PLASMINOGEN ACTIVATOR INHIBITOR IN ESSENTIAL THROMBOCYTHEMIA AND POLYCYTHEMIA VERA

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A hypercoagulability state exits in patients with essential thrombocythemia (ET) and polycythemia vera (PV). *in vitro* and *in vivo* data demonstrated a reactive release from vascular wall of tissue plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1) after increase in thrombin and t-PA concentration, respectively. Elevated plasma levels of t-PA and PAI-1 are well established thrombotic risk factor. However, few studies have concentrated on the correlation between hypercoagulability and fibrinolytic system in these malignancies. Prothrombin fragment F1+2 (F1+2), marker of thrombin generation, t-PA, activation marker of fibrinolysis, PAI-1 specific plasminogen activator inhibitor and d-dimer (DD), a product of fibrinolysis, were investigated in patients with ET and PV. As PAI-1 is abundant in platelet α-granules, we also measured b-thromboglobulin (b-TG) and platelet factor 4 (PF4) and platelets. We included 44 patients, 22 ET (5 men and 17 women, mean age 55 years) and 22 PV (17 men and 5 women, mean age 60 years) who fulfilled PVSG. The mean duration of disease was 4.5 years. Of 44 patients, 27 (13 ET, 14 PV) received hydroxyurea, 2 ET were on interferon-α, 5 ET were on anagrelide hydrochloride, 8 PV underwent phlebotomy and 2 ET not receiving any cytoreduction. All patients were on antiplatelets. None of studied patients had thrombotic risk factors. t-PA, PAI-1, F1+2, b-TG and PF4 were assayed by ELISA and DD by immunoturbidimetric latex agglutination. Platelets were determined by automated analyser. F1+2 (2.8±2.8 nmol/L vs 0,7±0.2 nmol/L) ($p=0.001$) was increased as well as t-PA and PAI-1 (112±67 ng/mL and 50±24 ng/mL, respectively, vs 9.6±2.4 ng/mL and 24±9 ng/mL, respectively) ($p=0.0001$ and $p=0.0001$, respectively), whereas DD was normal (157±125 ng/mL). All patients had elevated b-TG and PF4 (352±608 IU/ml and 129±49 IU/ml, respectively vs 25±9 IU/ml and 5±2 IU/mL, respectively) ($p=0.001$ and $p<0.0001$, respectively) and normal platelets ($402±148×10^9$/L). We found a correlation between F1+2 and t-PA ($p<0.0001$) and an association between t-PA and PAI-1 ($p<0.0001$). No correlation there was between PAI-1 and b-TG and PF4 and platelets. Our findings suggest a relation between hypercoagulability and hyperfibrinolysis responsible for an hypofibrinolysis as reflected by high PAI-1 and normal DD. Additionally, it is hypothesized that increased PAI-1 is independent of platelet abnormalities suggesting that a vasculopathic hypo-fibrinolysis may be in patients with ET and PV.

0088

PLATELET, LEUKOCYTE AND COAGULATION ACTIVATION PATTERNS IN MYELOFIBROSIS WITH MYELOID METAPLASIA

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Background. Platelet and leukocyte activation has been demonstrated in polycythemia vera (PV) and essential thrombocythemia (ET), this being considered as a contributory mechanism to thrombosis. Data in patients with myelofibrosis with myeloid metaplasia (MMM) are, however, scarce. *Aims.* To assess the status of platelet, leukocyte and coagulation activation in patients with MMM. *Methods.* Platelet and leukocyte activation status, including platelet P-selectin expression (measured at baseline and after ADP, thrombin and arachidonic acid stimulation), platelet-neutrophil and platelet-monocyte complexes, and CD11b determination in the neutrophils and monocytes, was assessed by flow cytometry in 20 MMM patients and in 22 age- and sex-matched healthy individuals. JAK2 V617F mutational status and the markers of coagulation activation prothrombin fragment 1+2 and D-dimer were correlated with platelet and leukocyte activation. *Results.* As compared with the controls, patients with MMM showed significantly higher values of baseline P-selectin expression (6.1% vs. 3.3%, $p< 0.001$), platelet-neutrophil complexes (31% vs. 16%, $p= 0.01$), platelet-monocyte complexes (65% vs. 34%, $p<0.001$), and neutrophil and monocyte CD11b expression (461 + 354 vs. 246 + 112 MFI units, $p=0.03$; and 966 + 456 vs 536 + 269 MFI units, $p=0.002$). Significantly higher plasma levels of prothrombin fragment 1+2 were also found in MMM patients, being associated with higher CD11b expression.

No differences in platelet and leukocyte activation were observed according to the JAK2 status. *Interpretation and Conclusion.* Patients with myelofibrosis show platelet, leukocyte and coagulation activation patterns similar to those found in PV and ET.

0089

EPIGENETIC ALTERATIONS AND MUTATION OF JAK2 TYROSINE KINASE IN PATIENTS WITH BCR/ABL NEGATIVE MYELOPROLIFERATIVE DISORDERS

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Background. Bcr/abl negative myeloproliferative disorders (MPD) are a group of clonal stem cell diseases and comprise traditionally essential thrombocythemia (ET), polycythemia vera (PV) and myelofibrosis with myeloid metaplasia (MMM). The recent discovery of the autoactivating mutation with a V617F amino acid substitution in the JAK2 tyrosine kinase has been a great step in the understanding of the pathophysiology of MPD. However, this mutation is found only in about half of the patients with MPD. *Aims.* Hypermethylation of CpG islands within gene promoter associated regions is associated with transcriptional inactivation and represents an important mechanism of gene silencing in the pathogenesis of human cancer. In this study, we sought to determine the potential role of DNA methylation changes in the context of JAK2 mutation in MPD. *Methods.* We analysed the JAK2 mutational status by direct sequencing and the methylation patterns of 12 cancer-related genes by methylation specific polymerase chain reaction in bone marrow and blood specimens from 33 patients with MPD. Genes analysed were SOCS-1, E-cadherin, MGMT, TIMP-2, TIMP-3, p15, p16, p73, DAPK1, RASSF1, RAR_2 and MLH1. *Results.* The frequency of aberrant methylation was 4/22 for SOCS-1, 1/22 for p15, TIMP-2 and E-cad in patients with MMM, 1/7 for SOCS-1 and MGMT in PV and 1/4 for SOCS-1 and DAPK1 in ET. We detected at least one hypermethylated gene in 11/33 patient samples. The JAK2V617F mutation was found in 9/22 patients with MMM, 4/7 with PV and 1/4 with ET. Our data indicate that hypermethylation of tumour suppressor genes can be considered as a common phenomenon in bcr/abl negative MPD in addition to the JAK2V617F mutation. We found concomitant heterozygous mutation of JAK2 and hypermethylation of the cytokine regulator SOCS-1 in two patients. The cell adhesion gene E-cadherin was methylated in one patient with MMM with simultaneous heterozygous JAK2 mutation. One patient with PV was detected to carry both mutation of JAK2 and hypermethylation of SOCS-1. However, in most patient samples, we found either JAK2V617F somatic mutation without CpG island hypermethylation or altered methylation patterns without genetic aberration of JAK2. *Summary.* In conclusion, we detected in MPD, in addition to the recently discovered activating mutation of JAK2, CpG island hypermethylation of cancer-related genes, especially SOCS-1, a negative regulator of JAK2. These results suggest, that epigenetic changes may, in addition to the well defined JAK2 activating mutation, contribute to the pathogenesis of bcr/abl negative MPD and thus can be considered as a potential therapeutic target for demethylating agents.

0090

CLINICOPATHOLOGIC HETEROGENEITY OF CHRONIC HYPEREOSINOPHILIC SYNDROMES LONG-TERM EXPERIENCE ON 32 PATIENTS

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Background. Chronic hypereosinophilic syndromes (CHS) comprise a wide spectrum of indolent to aggressive diseases characterized by prolonged, unexplained hypereosinophilia.*Aims.* A study was planned to evaluate clinical and pathologic features in patients with chronic, non-reactive hypereosinophilia. Material: 32 patients (pts) observed between 1990-2005 with absolute eosinophilia count (AEC) of higher than 0.7×10^9/L for at least 3 months were included. *Results.* There were 13 males and 19 females, aged 18-76 yrs (median 52 yrs). The diagnosis was following: Hypereosinophilic Syndrome (HES, n=16), Chronic Idiopathic Eosinophilia (CIE, n=12), T-cell mediated HES (n=1), Chronic Eosinophilic Leukemia (CEL, n=1) and CEL PDGFRA+ (n=2). Organ involvement included: heart (n=9), lungs (n=5), spleen (n=7), liver (n=4), lymph nodes (n=4), skin (n=3), peripheral nerves (n=2) and gut (n=1). Median white blood cell (WBC) count at diagnosis was 12.6×10^9/L (range 5.2-81.6), with AEC of 4.32×10^9/L (0.9-32.9) and bone marrow eosinophilic infiltration of 30% (8.0-55.0). Median IgE level was 95.2 IU (range 0.1-13966), vitamin B12 concentration-250pg/ml (range 60-3359). Only one patient revealed a slight increase in spindle-shaped mast cells on bone marrow exam, but there were no c-kit D816V and FIP1L1-PDGFRA mutations in this case. On cytogenetic evaluation normal karyotype was present in 13/14. One patient with CD3+CD4+CD8- T-cells and TCR$\alpha\beta$ rearrangement showed t(6;11)(q21;q23). BCR/ABL was undetectable in 19/19. RT-PCR for FIP1L1-PDGFRA was detectable in 2 of 19 pts at diagnosis (11%). The first-line therapy consisting of steroids alone or in combination with hydroxyurea was initiated in 21, patient with T-cell mediated HES received CHOP regimen and one patient with CEL in accelerated phase was given induction therapy (HAR-hydroxyurea, adriamycin, ara-c). Majority of pts with HES and CIE responded prompt to low dose of prednisone (10-20 mg/day), but eosinophilia recurred shortly after prednisone tapering or discontinuation. Eight patients due to resistance to prior therapy were administered imatinib at initial dose of 100 mg daily. A complete remission was documented in 3/8 (37%), Two out of three, who achieved complete hematologic remission in a median time of 14 days (range 13-65), were FIP1L1-PDGFRA positive at diagnosis. One out of two FIP positive patients achieved molecular remission at six months. All these three patients attempted to discontinue imatinib, but relapsed promptly. Imatinib was resumed at 100 mg daily with rapid eosinophilia resolution. Patient with CEL underwent allogeneic bone marrow transplantation from his brother and currently is disease-free. Patient with T-cell mediated HES was performed autologous stem cell transplantation being in complete molecular remission while eosinophilia persisted. One patient developed pure red cell aplasia during the disease course and it results from the prior hydroxurea treatment. Current status of pts included to the study is following: complete remission in 17 pts, partial response 4 pts, non-responders- 10 pts, 1 death due to cardiac insufficiency. *Conclusions.* Our study showed that majority of pts with CHS has a benign disease course and steroids are sufficient to control the eosinophil count. We confirmed the high efficacy of low dose of imatinib in patients carrying the FIP1L1-PDGFRA mutation. Discontinuation both steroids and imatinib was followed by rapid eosinophilia recurrence.

0091

APPLICATION OF PRV-1 MRNA EXPRESSION LEVEL AND JAK2V617F MUTATION FOR DIFFERENTIAL DIAGNOSTICS BETWEEN POLYCYTHEMIA VERA AND SECONDARY ERYTHROCYTOSIS. INFLUENCE OF INTERFERON THERAPY ON PRV-1 EXPRESSION

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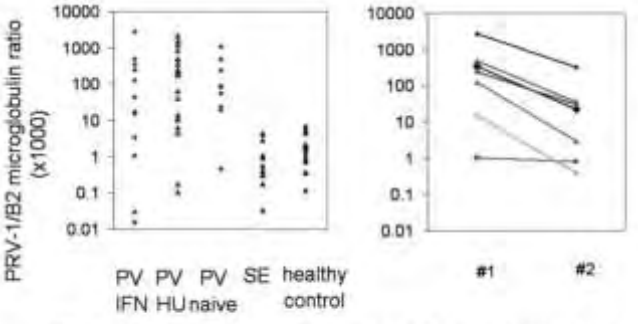
Background. Polycytemia vera (PV) is a clonal myeloproliferative disorder (MPD) lacking specific biological markers. Recent discovery of PRV-1 mRNA overexpression and Jak2V617F mutation in the majority of patients with PV can facilitate differential diagnosis between PV and non malignant disease - Secondary Erythrocytosis (SE). Furthermore, recently established influence of interferon (IFN) therapy on PRV-1 overexpression might reflect treatment efficiency. *Aims.* Confirm PRV-1 mRNA overxpression and Jak2 mutation in the group of patients diagnosed with PV and absence of these markers in the group of patients diagnosed with SE. Investigate influence of Interferon therapy on PRV-1 expression levels in patients with PV. *Methods.* We studied 46 patients (22 M/24 F) diagnosed with polycythemia vera based on PVSG criteria. For 39 patients diagnosis was confirmed by histological studies. Average patient age was 54 years, average time from diagnosis was 6.8 years. At the time of study out of 46 patients 9 were naïve and did not receive cytostatic treatment, 25 were pretreated with Hydroxyurea (HU) and 12 were pretreated with IFN. PRV-1 expression level was determined twice for 7 patients continuously receiving 3 millions ME daily 8 month after first analysis. Also we studied 14 patients with secondary erythrocytosis. Total duration of Interferon treatment for these patients was 23 month. Control group includes fifteen normal donors. PRV- 1 expression level was determined by reverse transcription and quantitative PCR (ICycler IQ, BioRad). Normalization to $\beta2$ microglobulin expression level was used for comparison between different samples. Jak2V617F mutation was determined by sequencing. *Results.* In the control group PRV-1 expression level was 1.17×10^{-4} -7.17×10^{-3}. PRV-1 mRNA overexpression was observed in 8 out of 9 naïve patients without cytostatic treatment, in 21 out of 25 patients pretreated with HU and in 8 out of 12 patients

pretreated with IFN. Overall we found PRV-1 overexpression in 37 out of 46 patients in the study (80%). We did not find PRV-1 overexpression in patients diagnosed with SE (Figure, left panel). Sequencing for determination of Jak -2 mutations was performed for 22 patients diagnosed with PV. From this group 5 were naïve, 9 *pretreated HU, 7* pretreated with IFN. Also 6 patients with SE and 3 healthy donors were examined. Mutation was found in all patients with PV and was not found in patients with SE and healthy donors. After 8 month of prolonging treatment with IFN PRV-1 overexpression level was decreased strongly (p=0.04) in all 7 examined patients (Figure, right panel). Six patients from this group archived remission at the time of second analysis. Criteria's of remission were reduction of platelet level down to 400-600×10^9/L, leukocytes to 10-12×10^9/L, and erythrocytes to 6×10^{12}/L.

Conclusions. We show high sensitivity specificity and utility of PRV-1 expression level and especially Jak2V617F mutation for differential diagnosis between PV and SE. Decrease of prv-1 expression levels in the group of patients receiving Interferon might be designated in the future as a molecular marker of treatment efficiency.



PRV-1 mRNA expression level from granulocytes of patients diagnosed with PV, SE and healthy donors. Patients with PV were naive or treated with HU or IFN. Right panel shows changes in PRV-1 expression of PV patients after 8 month of prolonged INF therapy

0092

INFLUENCE OF LONG TERM HYDROXYUREA TREATMENT ON PRV-1 EXPRESSION LEVEL IN POLYCYTHEMIA VERA PATIENTS

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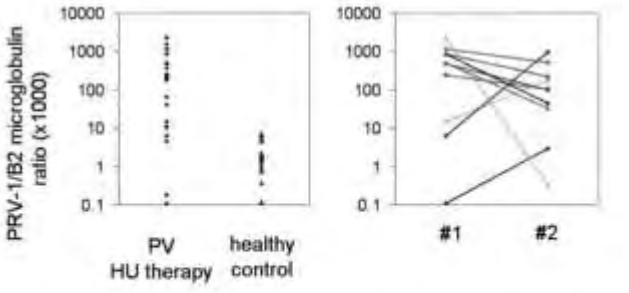
Background. Discovery of PRV-1 mRNA overexpression in patients with PV promises appearance of a specific marker for this disorder. Gene overexpression was found in 90% of naïve and treated patients. Further investigations revealed influence of therapy (for example Interferon treatment) on the PRV-1 expression level. However, instead of Interferon treatment, patients often receive Hydroxyurea (HU) as a primary treatment (first line treatment). *Aims.*Our goal was to investigate influence of long term HU treatment on PRV-1 expression level in the granulocytes of PV patients. *Methods.* We studied 25 patients diagnosed with polycythemia vera based on PVSG criteria. For 23 patients diagnosis was confirmed by histological studies. Average patient age was 58.8 years, average time from diagnosis was 7.8 years and duration of HU treatment was 3.7 years. Six patients achieved remission. Criteria's of remission were reduction of platelet level down to 400-600×10^9/L, leukocytes to 10-12×10^9/L, and erythrocytes to 6×10^{12}/L. PRV-1 expression level was determined twice for 10 patients continuously receiving 1 g HU daily 8 month after first analysis. Control group includes fifteen normal donors.PRV- 1 expression level was determined by reverse transcription and quantitative PCR (ICycler IQ, BioRad). Normalization to β2 microglobulin expression level was used for comparison between different samples. *Results.*In the control group PRV-1 expression level was 1.17×10^{-4}± 7.17×10^{-5}. We found PRV-1 mRNA hyperexpression in 21 of 25 (84%) patients (left panel of the figure). Alterations of PRV-1 expression for 10 patients in response to 8 month of HU therapy are shown in the right panel of the figure. We did not find any significant difference in changes of PRV-1 expression in response to therapy (p=0.07). In three patients with relatively low PRV-1 expression level before treatment PRV-1 expression was increased after treatment while in seven patients expression levels was decreased after treatment. *Conclusions.* We conclude that HU treatment does not normalize expression levels of PRV-1. Although not normalized, PRV-1 does decrease in the majority of patients; however this decrease is much slower than what is seen with Interferon therapy. Moreover, even for patients treated with HU for more than three years, PRV-1 expression remains high. Furthermore, elevated PRV-1 mRNA correlates with a low incidence of remission in the group of patients treated with HU.



Comparison of PRV-1 mRNA expression level from granulocytes of PV patients received prolonged HU treatment with healthy control. Right panel shows changes in PRV-1 expression of PV patients after 8 month of prolonged HU therapy

0093

V617F JAK2 MUTATION IN CHILDREN WITH ESSENTIAL THROMBOCYTHEMIA (ET)

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Background. The diagnosis of ET is usually made by excluding other primitive myeloproliferative disorders (MPD) and reactive thrombocytosis, since no specific biological marker is available. Recently, a somatic mutation V617F of janus kinasis 2 (Jak2) has been found in virtually all adults patients affected by polycythemia vera and in about 35-50% of ET patients. On the other hand no informations are available about the occurrence of Jak2 mutation in the rare cases of ET in children. *Aim.*To evaluate the occurrence of V617F Jak2 in pediatric ET. *Methods.* We searched V617F Jak2 mutation with sequencing test and allele-specific PCR, in 20 children (15 females and 5 males, median follow up 7.5 years) diagnosed to be affected by ET in agreement with the PVSG criteria and they were compared to 47 consecutive adult ET (36 females and 11 males, median follow-up 9.2 years) younger than 65. The comparison between categorical variables was performed by chi-square statistic test with Yates variable and p-value <0.05 were considered statistically significant. *Results.* Heterozigous V617F Jak2 was found in 4 children and 28 adults. The correlation between mutated Jak2 and thrombotic complications occurring both at diagnosis or during follow-up is summarized in the following Table: The occurrence of V617F Jak2 resulted significantly less frequent in children than in adults (p=0.0069) while the results on our adult patients are strongly consistent with the data reported in other papers. *Conclusions.* Recently, a link between age and V617F Jak2 mutation incidence has been surmised, possibly related to the influence of age on genetic instability. At present we cannot exclude that our children with ET will develop V617F Jak mutation. However, on the basis of the present data, we conclude that in ET, V617F Jak2 mutation is less frequent in children than in adults. The V617F Jak2 mutation is common both in pediatric and adult ET patients presenting with unusual thrombosis, namely sovra-hepatic, portal and cerebral veins thrombosis.

	Children 20 (15F/5M)			Adults 47 (36F/11M)		
	Total	Thrombosis	Not	Total	Thrombosis	Not
V617 Jak2	4 (20%)	1 (25%) 1 BC	3 (75%)	28 (60%)	10 (35.7%) 2 cerebral vein, 1 MI, 5 BC, 1 DVT, 1 PAD	18 (64.3%)
WT Jak2	16 (80%)	0 (0%)	11 (10%)	19 (40%)	2 (10.5%) (1 cerebral, 1 portal vein)	17 (89.5%)

BC= Budd Chiari, MI= Myocardial infarction, DVT= deep vein thrombosis, PAD= peripheral artery thrombosis

0094

PREVIOUS HISTORY OF THROMBOTIC COMPLICATION IS THE MAIN RISK FACTOR THAT INCREASES THE INCIDENCE OF THROMBOTIC EVENTS IN ESSENTIAL THROMBOCYTHEMIA PATIENTS

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Background and Aims. Thrombotic and hemorrhagic complications are the main causes of morbidity in essential thrombocythemia (ET). We investigated the clinical and laboratory characteristics associated with the occurrence of these events, with the aim of identifying subgroups of patients who could benefit from antiaggregant and/or cytoreductive therapy. *Methods.* 306 consecutive ET patients followed between January 1979 and December 2002 (median age 58 years, male/female ratio 0.55, median follow-up 96 months) were included in this study. In order to identify the possible predictive factors of thrombotic risk, the following variables were considered: age, gender, platelet count at diagnosis and at the time of thrombotic event, previous history of thrombotic and/or hemorrhagic complications, disease duration and cardiovascular risk factors (arterial hypertension, hypercholesterolemia, diabetes, smoking, obesity and a familial history of thrombosis). *Results.* 46 patients (15%) experienced major thrombotic complications during the course of disease. Major thrombotic complications included stroke, transient ischemic attack, myocardial infarction, angina pectoris, peripheral arterial thrombosis, retinal artery occlusion, deep venous thrombosis and pulmonary embolism. Age, gender, platelet count at diagnosis and at the time of thrombotic event, and disease duration did not appear in our series to significantly increase the incidence of thrombotic complications. These events occurred in 26/64 (40.6%) patients with a previous history of thrombosis and in 20/242 (8.3%) without a previous history of thrombosis ($p<0.0001$ Fisher's exact test, odd ratio 7.6). When patients with no previous history of thrombosis were stratified according to the number of cardiovascular risk factors (none vs one vs more than one) we observed a significant correlation with the occurrence of thrombotic events ($p<0.05$). 31 patients (10%) experienced major hemorrhagic complications, mainly gastrointestinal tract bleeding; three of them had a positive and 28 a negative history of hemorrhagic events ($p=0.052$). Major bleeding was defined as an event that threatened life or organ function, or required a transfusion of red blood cells. *Conclusions.* This study was based on a large cohort of patients followed for many years at a single institution and confirmed that a previous history of thrombosis is the main risk factor for developing further thrombotic events during the follow up. Age and platelet count, generally accepted as very important risk factors for thrombosis, did not appear in our series associated with an increased risk for thrombosis. Asymptomatic patients with a negative history of thrombosis and without any cardiovascular risk factors can be considered at *low risk* and therefore should not be considered for treatment - regardless of platelet count, age, sex and the disease duration. For patients with a negative history of thrombosis, but with one or more cardiovascular risk factors, it is essential to correct them and further evaluate the opportuneness of an antiaggregant and/or cytoreductive therapy.

0095

RATIONALE AND DESIGN OF A DOUBLE-BLIND, PLACEBO-CONTROLLED TRIAL TO EVALUATE THE CORTICOSTEROID-SPARING EFFECTS OF ANTI-IL5 MONOCLONAL ANTIBODY (MEPOLIZUMAB) IN SUBJECTS WITH HYPEREOSINOPHILIC SYNDROME

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Background. Hypereosinophilic syndrome (HES) comprises a group of rare hematological disorders characterized by sustained eosinophil overproduction. Clinical manifestations result from damage to multiple organs associated with local release of toxic granule products by infiltrating eosinophils. Management is currently based on chronic corticosteroid, interferon-α, and/or cytotoxic therapies, each of which is associated with significant toxicity and tolerability issues. Mepolizumab is a humanized anti-IL-5 monoclonal antibody that blocks the actions of IL-5, the major hematopoietin responsible for eosinophil production, differentiation, and survival. Preliminary data from a small number of patients with HES, asthma, and atopic dermatitis indicated that treatment with intravenous mepolizumab was associated with reduced blood eosinophils and was well-tolerated. Due to the variability in clinical presentation and lack of validated disease indices for HES, clinical parameters alone may not provide a sensitive and precise measure of drug efficacy. However, the ability of a treatment to enable corticosteroid sparing would provide clinically meaningful evidence of efficacy. Here we describe the design and conduct of a trial currently underway to evaluate the steroid-sparing effects of mepolizumab treatment in patients with HES. *Aims.* The primary objective of this ongoing study is to assess the effect of mepolizumab versus placebo on reducing corticosteroid requirements in patients with HES requiring 20-60 mg/day of prednisone to maintain eosinophils at <1500cells/μL. The primary endpoint is the proportion of subjects requiring ≥10 mg/day prednisone for at least 8 consecutive weeks during the 32 week treatment period. *Methods.* This multicentre (30 sites worldwide), randomized, double-blind, placebo-controlled, parallel-group study recruited patients 18'85 years of age with HES (blood eosinophil count >1500/μL for ≥6 months with evidence of organ involvement or dysfunction related to eosinophilia, without any other cause of eosinophilia), who were steroid-responsive and tested negative for the FIP1L1-PDGFRα gene rearrangement. Eligible patients were stabilized on prednisone monotherapy (20-60 mg/day) over a 6-week run-in period and then randomized 1:1 to receive intravenous mepolizumab 750 mg or saline (placebo) every 4 weeks. Prednisone was tapered at weekly intervals following the first infusion according to a pre-specified algorithm. HES-related end-organ involvement was monitored using cutaneous assessments, echocardiograms, computed tomography scans of the lung, abdomen and maxillary sinus, pulmonary function tests, and esophagogastroduodenoscopy. Patients' perceptions of HES symptom bother, health status, and limitations of daily living were determined using quality of life questionnaires. Blood samples were collected to characterize mepolizumab pharmacokinetics. Subjects completing the trial, or who withdraw due to lack of efficacy, could enter an open-label extension study to evaluate the long-term safety, efficacy and optimal dosing frequency of intravenous mepolizumab. *Results.* This trial, initiated in March 2004, was fully enrolled by May 2005 with 85 patients started on study medication. *Summary/Conclusions.* This ongoing study is the largest trial to be conducted to date in patients with HES, and the only placebo-controlled trial in this population. Results from this study will provide clinically important information on the treatment of HES with mepolizumab, and enable better understanding of this rare condition.

0096

FIP1L1-PDGFRA+ HYPEREOSINOPHILIA: HOW MANY DISEASES ?

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Background. The Hypereosinophilic Syndrome (HES) has remained for a long time a problematic diagnosis. WHO criteria relies on identification of rare signs of clonality which allow differential diagnosis between chronic eosinophilic leukaemia (CEL) and true idiopathic states (HES). Recently, a new mechanism of mutation was described: a cryptic interstitial microdeletion at chromosome band 4q12 generating a FIP1L1-PDGFRA (F/P) fusion gene (FG). According to the WHO guidelines, this clonal abnormality has been proposed as a new surrogate marker for CEL. Subsequently, the F/P FG was reported in patients with hypereosinophilia and atypical bone marrow (BM) mast cells (MC), suggesting an new hypothetical systemic mast cell disorder with hypereosinophilia subgroup (F/P+ SMCD-eos). Unfortunately, these SMCD-eos diagnoses were mainly based on histologic criteria (i.e., the major and the first minor WHO criteria) which are essentially subjective. The leukemic stem cell where the F/P deletion arises as well as the specificity of the loosely aggregates of tryptase positive mast cells in CEL remain to be identified before the relationship between these two clinical entities could be deciphered. In this regard, WHO rules for CEL and SMCD-eos differential diagnosis may deserve to be updated. *Aims.* We stressed out the potential subjective bias in WHO criteria interpretation and questioned the relationship between FIP1L1-PDGFRA+ CEL and SMCD-eos.

Specificity of tryptase expression in the context of hypereosinophilic diseases was assessed at the protein and mRNA level.

Table 1. Clinical, cytogenetics and molecular characteristics of the patients.

Cases	*Patient # 1*	*Patient # 2*	*Patient # 3*	*Patient # 4*
Age (year)/sez	39M	73/M	41/M	40/M
Dominant symptoms	Thoracic and back pain	Lab discovery	Cough, weight loss, asthenia	Cough, dyspnes, weight, loss pruritus
Splenomegaly	No	No	Yes	Yes
Cardiac involvement	Mitral valve disease	No	No	No
Other organ involvement	Pulmonary	No	No	No
Therapy before imatinib mesylate	Steroids	Steroids	NO	Steroids, HU, IFNα
Serum Vit B12 (pg/mL)	> 2000	841	>2000	>2000
Serum IgE (UI/mL)	14,1	47,9	22	N.A.
Serum tryptase	10,2	9,5	74,9	N.D.
Hgb, g/dL	11,5	10,4	10,1	12,6*
WBC×10^9/L	15,8	12,7	58,1	27,3*
ANC, ×10^9/L	1,81	7,58	24,40	3,52*
AEC ×10^9/L	8,9	2,4	27,3	19,1*
REC (%)	59	39	47	70*
Platelet ×10^9/L	194	314	27	116*
BM blastosis (%)	< 5	2	< 5	1,1**
BM eosinophils (%)	40	19	38	5**
Myelofibrosis°	-	N.D.	+++	N.D.
BM tryptase +MCs (IHC)	+	N.D.	+++	N.D.
Multifocal dense infiltrates$	-	N.D.	-	N.D.
> 25% of MCs are spindie-shaped	-	N.D.	-	N.D.
CD117+ and CD2+ or CD25+ BM MC	N.D.	N.D.	CD 117±; CD2-; CD25-	N.D.
Karyotype	46,XY (23)	46, XY (21)	46, XX (42)	46, Xy (42)
TCRγ gene rearrangement (PCR)	Oligoclonal	Oligoclonal	Polyclonal	N.D.
BCR-ABL fusion gene transcript (RT-PCR)	-	-	-	-
FIP1L1-PDGFRA	FIP1L1 13 -	FIP1L1 e 11-	FIP1L1 e 16-	FIP1L1 e 13-
fusion gene (RT-PCR)	PDGFRA e 12	PDGFRA e 12	PDGFRA e 12	PDGFRA e 12
CHIC 2 deletion/ISH (% abnormal nuclei)	70	24	76	0
Asp 816Val c-kit mutation	-	-	-	-
JAK2 V617F	wt	wt	wt	wt

M: male; F: female; HU hydroxyurea; IFNα: interferon alpha; BM: bone marrow; AEC: absolute cosmopol count ; REC: relative eosinoph count; MC mast cells; -: absent; ++: present; +++: marked; NA: not available; ND:not done; PCR polymerase chain reaction; RT: reverse transcription; I: intron; e: exon; wt: wild type.

*Collected after administration of steroids and HU and ** after IFNα

°Foot staining or hemotoxylin-eosyn coloration

$Must cells infiltrates ar rated as multifocal and dense (+) wen Z 15 aggregate cells ar present in multiple areas of the BM trephine biopsy

Methods. We described four patients initially diagnoses with HES according to the WHO guidelines, draw special attention to the disease clinico-biological characteristics and highlighted the WHO guidelines ambiguities. Tryptase expression was further assessed at the protein (UniCAP Tryptase immunoassay) and RNA levels (quantitative RT-PCR). *Results.* The F/P fusion gene was identified by FISH and RT-PCR in all our HES patients and, according to the WHO classification, a FIP1L1-PDGFRA+ CEL diagnosis could be made. However SMCD diagnosis criteria could not be ruled out without any ambiguity. Serum tryptase level was elevated in one case. Bone marrow trephine biopsies available for two cases were subjected to tryptase IHC. Whereas a diffuse interstitial infiltrate of loosely scattered tryptase positive MC was observed, there were no multifocal and dense (i.e., 15 or more mast cells in agregates) infiltrates. Specificity of tryptase (TPSB2) messenger RNA quantification was evaluated in control cases with hypereosinophilic diseases and in our patients. TPSB2 expression was detected in mastocytosis and F/P+ CEL but, miss underscoring its specificity, also in F/P- CEL and other pathologies with primary or reactive hypereosinophilia (HE). *Conclusions.* Our results confirm that molecular tools are mandatory for accurate F/P+ HE differential diagnosis, treatment option and follow-up. Positive tryptase IHC seems to be insufficient to advocate for F/P+ SMCD-eos diagnosis according to the WHO criteria for SMCD diagnosis. Moreover tryptase mRNA expression is not restricted to SM but could also be observed in reactive states as well as with CEL. Additional investigations are required to define the exact origin of the disease, better demonstrate F/P+ mast cells involvement as well as to introduce a WHO hypereosinophilic's syndrome criteria diagnosis update.

0097

VEGF SERUM LEVELS AND VEGF BONE MARROW IMMUNOHISTOCHEMICAL EXPRESSION IN CHRONIC MYELOPROLIFERATIVE DISORDERS

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Background. Angiogenesis plays a significant role in the pathogenesis and progression of chronic myeloproliferative diseases (cMPD). Vascular endothelial growth factor (VEGF), the most potent direct-acting angiogenic factor known, has been identified as a major cytokine underling pathological angiogenesis and hematopoiesis. *Aims.* To evaluate VEGF serum levels and VEGF bone marrow immunohistochemical expression in cMPD patients. To further analyze for possible correlations, between VEGF serum levels and other histopathological parameters, including VEGF immunohistochemical expression and bone marrow microvessel density (MVD). *Methods.* We evaluated serum levels of vascular endothelial growth factor (VEGF) in 73 patients with cMPD (25 patients with myelofibrosis with myeloid metaplasia (MMM), 40 with essential thrombocythaemia (ET) and 8 with polycythaemia vera (PV). Twenty seven healthy subjects' age and sex matched to the patient cohort were also included in the study. Moreover, immunohistochemical expression in bone marrow specimens in 25 MMM and 36 ET patients was studied. *Results.* We found that serum VEGF levels were significantly increased in cMPD patients comparing to controls (all p values were ≤0.012). Interestingly, bone marrow VEGF immunohistochemical expression was not increased in patients compared with controls. A high level of VEGF protein was detected in erythroid cells in patients and controls. By contrast myelocytes and megakaryocytes exhibited a variable expression of VEGF protein only in patients. Osteoblasts, osteoclasts and fibroblasts also were labeled by the anti-VEGF antibody in MMM patients. Serum VEGF levels were correlated with the percentage of bone marrow VEGF positive cells in all patients (r=0.53 p=0.001), as well as in MMM and ET separately compared with control group (r=0.68 p=0.001 and r=0.58 p=0.001 respectively). Serum VEGF levels were also correlated with MVD in all patients and in MMM patients separately (r=0.4 p=0.0013 and r=0.58 p=0.007 respectively). Bone marrow VEGF expression was not correlated with MVD. *Conclusions.* These data suggest a model during the course of cMPD, in which amplified secretion of VEGF from bone marrow cells and not increased numbers of VEGF secreting cells, produce increased levels of VEGF contributing to the disease phenotype. Anti-VEGF therapies may have a role in cMPD.

0098

ARRAY COMPARATIVE GENOMIC HYBRIDIZATION REVEALS AN ABSENCE OF RECURRENT GENOMIC COPY NUMBER CHANGES IN ESSENTIAL THROMBOCYTHEMIA

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Background. Essential thrombocythemia (ET) is a chronic myeloproliferative disorder characterized by an hyperplasia of the megakaryocytic cells in bone marrow resulting in a persistent increase of the platelet number in peripheral blood. Only about 5% of ET patients show chromosomally abnormal clones by conventional cytogenetics (CC), and about 15% of cases present chromosomal aberrations when FISH probes such as centromeric probes for chromosomes 8 and 9, and locus specific probes for 13q14 and 20q12 regions are applied. Thinking on the possibility of the existence of small DNA gains or loses not detected by the previously applied cytogenetic techniques, due to their low resolution (10 Mb for CC) or due to the restriction of the analyzed regions (only four regions of all the genome analyzed by FISH), array comparative genomic hybridization (aCGH) appeared to be a potentially useful new technology. *Aims.* The aim of the present study was to perform aCGH in order to identify at high resolution regions of DNA copy number changes associated to ET. *Patients and Methods.* Twenty cases diagnosed of ET according to the PVSG criteria (1997) and who had never received cytolytic treatment were studied. Conventional cytogenetics was performed in 17 cases, and 13 of them showed a normal karyotype while in 4 cases no metaphases were available. In five cases, FISH with BAC probes for the PRV-1, TPO and c-MPL genes was done, but no genetic abnormalities were detected. Genomic DNA was extracted from fresh frozen granulocytes, and pools of karyotypically normal males and females DNAs were used as controls. Forward and reverse hybridiza-

tions were performed in test and control samples using the Spectral Chip 2600TM (Spectral Genomics), an array consisting on 2,621 BAC clones at an average of 1-2 Mbp resolution, according the manufacturer's specifications. Fluorescent images were obtained using an Agilent G2565BA scanner and quantified using GenePix 6.0 software (Axon, Molecular Devices) using the irregular feature finding option. Extracted raw data was filtered and normalized using Bacanal (Lozano *et al.,* unpublished), an in house web server implementation of the Limma package developed within the Bioconductor project in the R statistical programming environment. *Results.* Among the 20 analyzed patients, in two cases a genomic copy number change was detected. Case 2 showed a gain of 3p24-p24.3 (RP11-245E5, RP11-208G16) and case 6 presented a gain of 8p23.2 (RP11-121F7, RP11-11P7), a region that encodes the CSMD1 gene. In addition, two patients (cases 11 and 12) showed variation copy number polymorphisms in 16p11.1-p11.2 (RP11-488I20, variation 0196 and RP11-80F22, variation 0197) and in 2q37.3 (RP5-1011017, variation 0032 where FLJ40712 and FLJ41327 are located and CTB-172I13), respectively. The gains and losses detected in these patients were not detected in the remaining patients. *Comments.* Array CGH reveals an absence of recurrent genomic copy number changes in essential thrombocythemia. FISH studies with the affected BAC clones will be performed to confirm these *Results. Acknowledgments.* Grants FIS PI030345, C03/07 and C03/10 from the Spanish Ministry of Health.

0099

THE INCIDENCE OF DEL20Q12 BY INTERPHASE FLUORESCENCE *IN SITU* HYBRIDIZATION (I-FISH) IN PATIENTS WITH CHRONIC MYELOPROLIFERATIVE DISORDERS

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Backgroud. Chronic Myeloproliferative Disorders (CMPD), including Polycythemia Vera (PV), Essential Thrombocythemia (ET), Idiopathic Myelofibrosis (IMF) and Unclassified CMPD, are acquired diseases of the hematopoietic stem cell, characterized by clonal proliferation of one or more cell lineages. Chromosomal abnormalities have been reported in less than 20% of CMPD patients at the time of diagnosis and in higher frequencies during the course of the disease.

*Aim.*The aim of the present study was to determine by I-FISH the incidence of 20q12 deletion (del20q12) (D20S108) in bone marrow samples from CMPD patients either at diagnosis or during the course of the disease and to assess its clinical utility.

Patients and Methods. Eighty four samples from 38 men and 40 women with median age 63 (range 17-84) years were studied using I-FISH, utilizing a locus specific probe for 20q12 (D20S108). 38 patients were diagnosed with ET, 34 with PV, 3 with IMF and 3 with unclassified CMPD. Bone marrow samples from 12 healthy volunteers were used as assay validation controls. 31 samples were studied at diagnosis, whereas 53 during the course of the disease. The median duration of the disease at the time of analysis was 51 (range 1-192) months. Most of the patients (40/53) were treated with hydroxyurea either alone or in combination with aspirin, interferon or anagrelide for a median period of 55 (range 2-217) months. *Results.* The del20q12 was detected in 19 out of 84 samples (23%). The chromosomal aberration was revealed in 7/34 (21%) PV patients, in 9/38 (24%) ET patients and in 2/3 (67%) Unclassified CMPD. Sequential I-FISH studies were performed in six patients. In one PV patient, although the initial study was normal, the del20q12 was developed after a period of 21 months. The del20q12 was significantly associated with treatment failure (p=0.012) and inferior outcome of the disease. The five years survival without disease progression (myelofibrosis, secondary leukemia or myelodysplastic syndromes and death) was 62±15 months vs 89±5 months in patients with or without del20q12 respectively. Thus, patients with del20q12 were associated with significant increased odds of having disease progression (OR=3.1, 95% CI=1.1-9.0, p=0.037). Nevertheless, there was no correlation between the presence of del20q12 and age, sex, therapy duration as well as the interval between the presentation of the disease and I-FISH analysis. *Conclusion.* Our experience, in concordance with other studies, showed that the del20q12 can be revealed by I-FISH in approximately 20% of CMPD patients. Poor prognosis and treatment failure were statistically associated with the del20q12. Larger and prospective series are needed to ascertain the possible clinical implications of del20q12 in CMPD patients.

0100

ABNORMAL EXPRESSION OF THE WAP FAMILY SLPI GENE IN PATIENTS WITH CHRONIC MYELOPROLIFERATIVE DISORDERS

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Background. Abnormal expression of several protease inhibitors has been demonstrated to occur in granulocytes from patients with polycythemia vera (PV) using gene expression profiling (Pellagatti *et al.* Cancer Res, 2003). Among these, SLPI, a member of the WAP (whey-acidic-protein) family that has been involved in cell cycle, apoptosis regulation, and the expression of a neoplastic phenotype in several solid tumors was among mostly upregulated genes. Abnormalities of 20(q11;13), where SLPI maps, have been reported in 10-25% of patients with idiopathic myelofibrosis (IM). AIMS The aim of the study was to evaluate whether abnormal expression of SLPI characterizes patients with MPD, and whether it might help in distinguishing between the different clinical entities. *Methods.* We studied 102 patients with MPD (23 PV, 58 ET, 21 IM) diagnosed according to WHO criteria, and 39 blood donors as healthy controls. Expression levels of SLPI and PRV-1 were determined by a quantitative TaqMan RT-PCR using granulocyte RNA and GAPDH as the housekeeping gene. In healthy blood donors the mean CT ratio for SLPI was 7.09±1.21 (range, 5.23-9.03); SLPI was considered overexpressed when CT ratio was equal or lower than 4.67. *Results.* The mean expression level of SLPI in the whole MPD population (4.3±1.8) was significantly higher than in healthy subjects (p <0.0001). There was a progressive increase in SLPI overexpression in patients with IM (3,06±1.60) to PV (3,81±0.92; p=0.06) and to ET patients (4,98±1.85, p<0.0001); levels in ET patients were also significantly lower than in PV (p=0.005). By using the 4.67 cut-off, 66/102 patients had SLPI overexpression (65%), accounting for 96% (22/23), 43% (25/58) and 90% (19/21) of PV, ET and IM patients, respectively. The abnormal expression of SLPI correlated with the JAK2 mutational status: SLPI ratio was 3.16±1.62 in homozygotes, 4.53±1.78 in heterozygotes and 4.36±1.83 in wild-type patients; the difference between homozygote and heterozygote patients was statistically significant (p=0.02). There was no significant correlation between the overexpression of SLPI and that of PRV-1. To evaluate whether SLPI expression was modulated by cytokine exposure, as it has been shown for PRV1, we measured changes in SLPI mRNA levels after *in vitro* granulocyte activation with different cytokines such as G-CSF, IL8, IL3, IL11, IFN-γ and TNF-α. There was no significant modulation of SLPI unlike PRV1, that was induced by G-CSF exposure. SLPI plasma levels were measured by an ELISA assay; the mean plasma levels in MPD patients were significantly higher than in controls (64 ng/mL, range 48-86, and 39 ng/mL, range 29-51, respectively). *Conclusions.* In this study we have identified abnormal expression of SLPI gene as a novel molecular marker of MPD, that is also associated with raised plasma protein levels; there was also a disease-specific pattern of overexpression among IM, PV, and ET patients. The role of abnormal expression of SLPI in disease pathogenesis remains to be established.

0101

CONGENITAL ERYTHROCYTOSIS AND POLYCYTHEMIA VERA IN CHILDREN AND ADOLESCENTS - AN ONLINE DATABASE FOR REGISTRATION OF PATIENTS AND DATA COLLECTION

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Congenital erythrocytosis and Polycythemia Vera (PV) in children and adolescents represent rare and heterogeneous clinical entities. The current knowledge on the clinical presentation, laboratory investigation, as well as on the evolution and treatment of these disorders is sparse. *Aim.*In order to better characterize congenital primary and secondary erythrocytosis and PV in young patients, we developed an online computerized database for systematic registration of patients' data. *Material and Methods.* Patients at any age with apparently congenital erythrocytosis and patients younger than 20 years with PV. Neonatal polyglobuly will not be included. Selected patients with acquired secondary erythrocytosis can be followed as observational patients and may be included in the evaluation of particular aspects of the study. Diagnostic guidelines for apparently congenital erythrocytosis include the exclusion of potential underlying cardiac, pulmonary and renal disorders; the assess-

ment of diagnostic parameters characterizing polycythemia vera, e.g. the identification and allele specific examination of JAK2 mutation V617F and PRV-1-mRNA quantification; the screening for hemoglobin variants with high oxygen affinity and for familial 2,3-BPG deficiencies; the molecular analysis of factors related to disorders of erythropoietin synthesis (e.g. von-Hippel-Lindau gene mutations) and signaling (e.g. erythropoietin-receptor mutations). The database is located on www.erythrocytosis.org and can be accessed by any physician after online registration. Patients' identification is codified. Registration and data collection, after patient informed consent, include the clinical and family history, evolution, complications and treatment and the hematological, biochemical and molecular biology studies. At any time data can be updated and follow-up information can be introduced. Patient's family members can be genealogically connected. Among other potentialities, the database is prepared to generate statistical information, query based data exports in standard formats and dynamical patients profiles ready for checking the database for matches. Discussion involving registered doctors is a simple procedure. *Conclusion.* This European online database will be a powerful instrument to obtain systematic data on the clinical presentation, on results of various diagnostic procedures and the clinical evolution of patients with congenital primary and secondary erythrocytosis and of young patients with PV.

0102

ERYTHROCYTOSIS AND THROMBOCYTOSIS IN CHILDHOOD: THE EXPERIENCE OF A SINGLE CENTRE

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Background. Erythrocytosis (E) and thrombocytosis (T) in childhood are mainly secondary forms and result from several causes. The diagnosis of Polycythemia Vera (PV) and Essential Thrombocythemia (ET) can often be difficult and currently relies on clinical and biological criteria defined by WHO. Recently, an activating somatic point mutation of Jak2 has been described in the vast majority of patients with PV as well in subsets of patients with ET. Presence of this mutation is highly correlated with PRV-1 over-expression, another molecular marker of MPD. *Aims.* The aims of this report is to describe the clinical, biological and molecular features of 24 paediatric patients affected by E or T, focusing on primary forms. *Methods.* We conducted a retrospective study on all patients affected by E or T (including criteria: Hct> +2DS of the expected value or platelet count ≥ 1×10^6/mmc) referred to our Centre between 1st January 2000 and 31st December 2005. *Results.* Thirteen patients with E and eleven patients with T (M/F: 10/3 and 4/7; median age at diagnosis: 88 and 14 months, respectively) were investigated. 4/13 E resulted secondary to congenital heart disease, 1/13 secondary to persistent obstructive sleep apnea, 2/13 familiar forms and 6/13 primary erythrocythosis (PE). Only 1 among 6 PE was diagnosed as PV according to the WHO criteria; he showed thrombotic complications (a cerebral ischemia and a splenic infarction) and is currently treated with oral anticoagulant therapy, low-dose aspirin, hydroxyurea and regularly undergoes to phlebotomy. Reactive thrombocytosis (RT) was diagnosed in 6 out 11 T, associated with bacterial or viral infections (mean duration = 4,4 months) while ET was diagnosed in 5 (mean duration = 45 months) according to WHO criteria (see clinical features of PE and ET in Table 1).

	pt	s	Diagnosis age (y,m)	Hb (g%dL)	Hct (%)	spleno-megaly	EPO	EEC	molecular markers	treatment
PE	1	M	16,5	16	49	↑	N	A	none	no
	2	M	14,3	16,9	50,6	none	N	A	none	Aspirin
	3	M	5,6	22	62,8	↑	N	A	none	Aspirin, P
	4	M	16,6	18,1	51,3	none	N	A	none	P
	5	F	11	15,9	49	↑	N	A	↑PRV-1	P
	6 (PV)	M	13,5	16,8	52,8	↑↑↑	↓	+	↑PRV-1 V617F	Aspirin, Warfarin, Hydroxurea, P

	pt	s	Diagnosis age (y,m)	Diagnosis platelet (10^3/mmc)	spleno-megaly	molecular markers	treatment
ET	1	F	1,2	1014	none	none	Aspirin
	2	F	8	1043	↑↑	none	Aspirin, Hydroxurea, Anagrelide
	3	M	3,8	1349	↑	none	Aspirin, Hydroxurea, Anagrelide
	4	F	7,2	1937	↑	none	Aspirin, Hydroxurea
	5	M	6,4	1000	↑	none	Aspirin

Table 1. Legend: PE=Primary Erythrocytosis; ET=Essential Thrombocytemia N=Normal value; A=Absent; P=Phlebotomy

As far as the maximum platelet count is concerned no significant difference was found between ET and RT (mean = 1770±758 and $1510\pm391\times10^3$/mmc, respectively). On the contrary the duration of disease is significantly lower in RT ($p<0,001$). No thrombotic complications in T were documented; 3/6 RT and 5/5 ET treated with low-dose aspirin even if no prothrombotic factors were identified; 3/5 ET received also hydroxiurea, two of them received also anagrelide which was discontinued for important side effects. An isolated increase of PRV1 expression was observed in one PE and the presence of the missense mutation (V617F) in Jak2 gene associated with PRV1 over-expression was only found in the patient with PV. No molecular alterations were detected in TE patients. In all patients t(9;22) was excluded. Cytogenetic analysis did not show abnormalities. *Discussion.* PV and TE are extremely rare diseases in childhood and therefore few paediatric data on incidence, clinical significance and management are reported in literature. Regarding T, the maximum level of platelet count does not allow to differentiate between RT and TE while the platelet count normalization in few months suggests a secondary form. Molecular markers, useful in distinguishing between MPD and secondary forms, are rare in our patients. Collaborative studies are necessary to better define clinical features, diagnostic approach and therapeutic strategies.

0103

PREDICTIVE VALUE OF ALTERATIONS OF COAGULATION AND THE JAK2 MUTATION ON THE RISK OF THROMBOSIS IN ESSENTIAL THROMBOCYTHEMIA

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Background. Essential thrombocytemia (ET) is a heterogeneous disorder in which thromboembolism remains the major cause of morbidity and mortality. Previous studies have shown high frequency of thrombosis in patients with alterations associated to hipercoagulability or thrombophilia and more recently in patients with the JAK2 V617F mutation. *Aims.*First objective of this study was to study the presence of alterations of coagulation associated with risk of thrombosis in patients with ET and the second aim was to analyze the incidence of JAK2 mutation in those patients and its relation with haematological parameters and risk of thrombosis. *Methods*. We have studied 58 patients previously diagnosed of ET. Sex distribution was 25 males and 33 females. Age: ranged from 20 to 86 years (mean 59±15). Patients follow up was from six to 253 months (mean 89±54). In all patients, the presence of thrombophilia alterations was studied. The studies included: APTT, won Willebrand antigen, Protein C, Protein S, and Antithrombin III, lupic anticoagulant, anticardiolipin antibodies, Protein C Activated Resistance (PCAR), Factor V Leiden, Prothrombin 20210A and MTHFR mutation. JAK2 mutation was analyzed in 32 patients. The significance of this mutation was investigated in relation with, haemoglobin, leukocytes and platelets levels at diagnosis, and with the presentation of thrombosis. Statistical analysis was performed with SPSS software. *Results.* To 1ers objective: 17 patients (29%) develop thrombosis, 7 before diagnosis and 10 during evolution. 59% of thrombosis were in patients older than 60 years, mean age of thrombosis was 61 years old. 50% of patients with thrombosis showed high expression of von Willebrand antigen factor, Results in both groups of patients were (Mean±SE: 159_51 and 128_48) respectively. FV Leiden, P20210A, MTHFR mutations, low levels of protein C, S and Antithrombin III, PCAR, and presence of antiphosfolipid antibodies did not appear as independent risk factors. However, 83% of thrombosis happened in patients with one or two alterations in the study of thrombophilia. To 2º objective: JAK2 mutation was present in 56% of the ET patients. Patients with the mutation presented higher hematocrit values (44±5 vs 40±3; p=0.04), higher white blood cell counts 8951 vs 7304; p=0.05) and higher mean number of platelets (908.000 and 685.000 respectively; p=0.01). JAK2 Mutation was present in 5 from 8 patients with thrombosis and in 13 from 24 who do not develop thrombosis. *Conclusions.* 1. Thrombosis risk in ET is increased in patients with alterations of coagulation, however none of these alterations shows independent predictive value. 2. Results from our serie confirm the presence of JAK2 mutation in more than 50% of ET patients and shows relation of this mutation with hematocrit value and higher white cell and platelets counts. 3. Relation of JAK2 and thrombosis cannot be established at the present time out of prospective studies.

Acute myeloid leukemia I

0104

MOLECULAR PROFILING USING ARRAY-CGH AND GENE EXPRESSION PROFILING REVEALS NEW CANDIDATE GENES IN AML WITH NORMAL KARYOTYPE

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Clonal chromosome abnormalities represent one of the most important prognostic factors in adult acute myeloid leukemia (AML), and cytogenetic data are used for risk-adapted treatment strategies. By conventional cytogenetic analysis, approximately 50% of patients lack clonal chromosome aberrations, and normal cytogenetics are associated with an intermediate clinical outcome. This clinically heterogeneous group seems to be in part characterized by molecular markers, such as MLL, FLT3, CEBPA, and NPM1 mutations. In order to identify novel candidate regions of genomic imbalances, we applied comparative genomic hybridization to microarrays (array-CGH). Using this high-resolution genome-wide screening approach, we analyzed 49 normal karyotype AML cases characterized for the most common clinically relevant molecular markers (MLL-PTD n=13, FLT3-ITD n=7, FLT3-ITD/NPM1+ n=4, MLL-PTD/FLT3-ITD n=3, CEBPA+ n=12, CEBPA+/FLT3-ITD n=1; CEBPA+/NPM1+ n=1; no molecular markers n=8) with a microarray platform consisting of 2799 different BAC or PAC clones. In addition to known copy number polymorphisms in 5q11, 7q22, 7q35, 14q32, and 15q11, we were able to disclose copy number alterations (CNAs) in terms of gains in 9p, 11q, 13q and losses in 3p, 9p, 11q, 12p, 13q, and 16p. In a subset of cases we profiled global gene expression and the correlation of array-CGH findings with global gene expression profiles allowed the identification of candidate genes, e.g., FOXP1 and RYBP in 3p13 and MLL and DDX6 in 11q23. Furthermore, two-class supervised analyses using the significance analysis of microarrays (SAM) method identified for the MLL-PTD cases a gain of a single clone harbouring the MLL gene underlying the power of array-CGH detecting small genomic aberrations. While the significance of these findings, which were already in part validated using fluorescence in-situ hybridization (FISH), still remains to be determined, our preliminary results demonstrate the power and reliablity of this microarray-based technique allowing genome-wide screens of genomic imbalances. Furthermore, ongoing correlation of high-resolution genomic profiling with global gene expression studies will help to disclose pathways underlying normal karyotype AML, thereby leading to new insights of leukemogenesis.

0105

CO-EXPRESSION OF CD34, MDR1 AND BCRP INDICATES A CLINICALLY RESISTANT PHENOTYPE IN PATIENTS WITH ACUTE MYELOID LEUKEMIA OF OLDER AGE

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Clinical resistance to chemotherapy in acute myeloid leukemia (AML) is often associated with the expression of the multidrug resistance (MDR) proteins P-glycoprotein (P-gp), encoded by the MDR1/ABCB1 gene, multidrug resistant-related protein (MRP1/ABCC1), the lung resistance-related protein (LRP), or major vault protein (MVP), and the breast cancer resistance protein (BCRP/ABCG2). The clinical value of MDR1, MRP1, LRP/MVP and BCRP mRNA expression was prospectively studied in 154 newly diagnosed AML patients aged ≥ 60 years, who were treated in a randomized clinical trial. Expression of MDR1 and BCRP showed a negative while MRP1 and LRP showed a positive correlation with high white blood cell count (respectively $p<0.05$, $p<0.001$, $p<0.001$ and $p<0.001$). Higher BCRP mRNA was associated with secondary AML ($p<0.05$). A strong correlation between MDR1 and BCRP mRNA expression was observed ($p<0.001$), while also MRP1 and LRP mRNA co-expression was found ($p<0.001$). High MDR1 but not MRP1, LRP or BCRP mRNA expression was associated with a lower (CR) rate and with worse event-free survival (EFS) and overall survival (OS),using univariate analysis. Although CD34 expression overruled all other prognostic factors, co-expression of MDR1 and BCRP identified a clinically resistant subgroup of elderly AML patients with a low CR rate and poor EFS and OS (p-values respectively: 0.01, 0.01 and 0.05).

0106

REPLICATIVE SENESCENCE INDUCTION IN GOOD-RISK ACUTE MYELOID LEUKEMIA

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Immortal cell growth is considered the hallmark of tumor cells. In contrast, normal cells have a limited proliferative capacity of 40-60 cell divisions, also known as the Hayflick limit. The limited proliferative capacity of normal cells relates to gradual shortening of telomeric DNA as a consequence of the end-replication problem. Upon critical shortening of telomeric DNA, cells enter a non-replicative but viable state referred to as replicative senescence. These replicative senescent cells stain blue in a β-Galactosidase assay. Human fibroblast models have shown that escape from senescence results from loss of p53 and Rb function. Escape is associated with activation of a telomere maintenance mechanism, mostly reactivation of telomerase. Telomerase elongates telomeric DNA. High levels of telomerase, as observed in germ cells and most tumor cells, allow for immortal cell growth. Recently, we demonstrated relatively low levels of telomerase in AML patients with t(8;21) or inv(16) (Swiggers *et al.,* GCC 2006). Interestingly, levels of telomerase in these AML samples were similar to levels of telomerase in normal bone marrow progenitor cells. We hypothesized that these AML cells, where telomerase is not reactivated to high levels, may not have inactivated the senescence pathways that limit the proliferative capacity of normal cells. This hypothesis was addressed by studying AML patient samples with t(8;21), t(15;17) or inv(16) *in vitro* (long-term cell cultures in presence of growth factors) and *in vivo* (following transplantation in NOD-SCID mice and in patients at time of relapse) for cells with all characteristics of replicative senescence, i.e., viable, non-proliferating, blue-coloring in β-Gal assay, and critical short telomeres. AML cells with all characteristics of replicative senescence were clearly observed in AML samples with either t(8;21), t(15;17) or inv(16). Gradual telomere shortening was observed in these AML cells *in vitro* upon long-term culture, *in vivo* following transplantation in NOD-SCID mice and *in vivo* in patients at relapse, indicating that these AML cells do not have an adequate telomere maintenance mechanism. We included in the study a control group of AML that is characterized by telomerase reactivation to high levels (complex karyotype group, n=8). Cells with characteristics of replicative senescence were not induced *in vitro* or *in vivo* in any of the samples of this AML control group. We conclude that AML cells with t(8;21), t(15;17) or inv(16) are characterized by intact pathways that induce replicative senescence. Intact pathways that limit proliferative lifespan may be critical to the high cure rates following chemotherapy treatment of patients with good-risk AML.

0107

PROGNOSTIC IMPACT OF FLOW CYTOMETRICALLY DETERMINED MINIMAL RESIDUAL DISEASE IN PATIENTS WITH ACUTE MYELOID LEUKEMIA

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Background. Adaption of treatment in acute myeloid leukemia (AML) is based on individual risk profiles. Significant prognostic information may be provided by the level of minimal residual disease (MRD). *Aims.* To assess the prognostic impact of flow cytometrically quantified MRD levels in AML in a multivariate analysis. *Methods.* We applied multiparameter flow cytometry (triple staining) to highly sensitively quantify MRD in patients with AML. A total of 356 patients receiving standardized intensive antileukemic treatment was analyzed at different checkpoints: CP1 (up to day 28, n=156), CP2 (day 28-60, n=122), CP3 (day 61-120, n=195), CP4 (day 121-365, n=172), and CP5 (after day 365, n=73). Leukemic cells were identified by their individually defined leukemia-associated aberrant immunophenotypes (LAIPs). MRD levels were calculated as the logarithmic difference (LD) between LAIP-positive cells at diagnosis and LAIP-positive cells at follow-up assessment. *Results.* The median LD amounted to 2.18 (range, -0.14 to 4.20) at CP1, 2.31 (-0.03 to 4.17) at CP2, 2.49 (0.11 to 4.17) at CP3, 2.58 (-0.28 to 4.28) at CP4, and 2.87 (0.46 to 4.02) at CP5. A higher LD (continuous variable) was

related to a better event-free survival (EFS; CP1, p=0.0002; CP2, p=0.00001; CP3, p=0.0002; CP4, p<0.00001; CP5, p=0.00007) and to a better overall survival (OS; CP1, p=0.004; CP2, p=0.001; CP3, p=0.021; CP4, p=0.00006). Other parameters related to EFS and OS were age and cytogenetics in the present series. The prognostic impact of MRD levels on outcome was idenpendent of cytogenetics and age for EFS (CP2 to CP5) and OS (CP2 and CP4). MRD was the most important prognostic parameter at CP4 and CP5. Separation of patients into two groups, respectively, by the median LD resulted in significant differences in EFS at all CPs and in OS at CP1 to CP4. The largest difference was observed at CP4: median EFS, 57.1 vs. 13.7 months, p<0.00001; 3-year-OS, 95% vs. 65%, p=0.0003. *Summary*. A highly powerful and independent prognostic parameter is provided by the MRD levels determined by multiparameter flow cytometry which is applicable to the total of an AML population. It should be evaluated as a stratification parameter in clinical trials.

0108

PROGNOSTIC RELEVANCE OF FLT3-TKD MUTATIONS IN AML: THE COMBINATION MATTERS- AN ANALYSIS OF 3082 PATIENTS

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Background. FLT3-TKD mutations are located in the activation loop domain of the FLT3 gene and mostly represent point mutations in codon D835 or deletions of codon 836. They induce constitutive activation of the receptor tyrosine kinase and are supposed to represent gain-of-function mutations. These mutations have been described in AML previously, but in contrast to FLT3-LM/ITDs the distribution within AML subtypes and an impact on prognosis has not been reported, so far. *Methods.* We screened 3082 newly diagnosed patients with AML for this mutation by a LightCycler based melting curve analysis. Positive cases were further characterized by sequencing. *Results.* FLT3-TKD mutations were found in 147/3082 (4.8%) of all patients. Most patients revealed amino acid exchanges from D to Y at position 835 (68/137; 49.6%). Monoallelic mutations occurred in 118 cases (96.7%), whereas biallelic mutations accounted for 4 of 122 mutated cases (2.9%). In total, FLT3-TKD mutations occurred in nearly each cytogenetic subgroup. Similar to FLT3-LM/ITDs there was a significant correlation of FLT3-TKD mutations with t(15;17)/PML-RARA (10/130; 7.7%), whereas the incidence in normal karyotype was not significantly higher as compared to others. In addition, FLT3-TKD mutations were significantly underrepresented in complex aberrant karyotype (4/430; 0.9%), in 5q-/-5 (0/31; 0%), and in 7q-/-7 (1/59; 1.7%), i.e. in the prognostically unfavorable subtypes. The incidence was low in t(8;21)/AML1-ETO (2/88; 2.3%). With regard to morphology, FLT3-TKD mutations were overrepresented in the FAB subtype AML M3v (6/51; 11.8%) and in the FAB subtypes M1 (42/491, (6.3%)), M4 (39/484 (8.1%)), and M5b (15/114 (13.2%;)). In contrast to FLT3-LM, which are clearly associated with higher peripheral leucocytes, FLT3-TKD did not correlate with this parameter. A correlation of FLT3-TKD with other molecular mutations showed the highest incidence of FLT3-TKD mutations in cases with NPM1 (23/262; 8.8%), CEPBA (6/76; 7.9%), and NRAS mutations (6/78; 7.7%). FLT3-TKD in combination with FLT3-LM (17/594 patients; 2.9%) and KITD816 (1/44; 2.3%) was rare. In contrast to FLT3-LM which is known as strong negative prognostic parameter, overall survival (OS) and event free survival (EFS) were influenced neither in the total cohort (57 FLT3-TKD and 1623 FLT3-WT) nor in the normal karyotype group (97 FLT3-TKD and 764 FLT3-WT) . However, in the subgroup with t(15;17)/PML-RARA EFS was unfavourably influenced by FLT3-TKD mutations. With regard to other molecular mutations there was an additional unfavourable impact for FLT3-LM/TKD double mutated and MLL-PTD/TKD double mutated cases. In contrast, there was an additional favourable impact on EFS in NPM1 and in CEPBA. *Conclusion.* Thus, the FLT3-TKD mutations seem to have an unfavourable enhancement of unfavourable and a favourable intensification of prognostically favourable molecular mutations.

0109

EXPRESSION OF TUMOR-ASSOCIATED ANTIGENS IN ACUTE MYELOID LEUKEMIA AND THEIR CORRELATION WITH SURVIVAL

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Background. The expression of tumor-associated antigens (TAAs) might play a critical role in the control of minimal residual disease (MRD) in acute myeloid leukemia (AML). *Aims.* Here, we investigated whether TAAs were associated with clinical outcome in AML. *Methods.* A DNA-microarray analysis of 116 AML samples as well as ELISPOT, FACS and chromium release assays were performed to assess TAA-specific T cell responses in these patients. *Results.* A significant correlation of high mRNA expression of G250/CA9 with a longer overall survival (p=0.022), a trend for better outcome in patients with high expression levels of PRAME (p=0.103), and a hint for RHAMM/HMMR. In contrast, for other TAAs like WT1, TERT, PRTN3, BCL2, and LAMR1 we found no correlation with clinical outcome. Interestingly, the co-expression of RHAMM/HMMR, PRAME and G250/CA9 provided a favorable prognostic effect (p=0.005). We also observed specific T cell responses at high frequency for these antigens. Positive immune reactions were detected in 8/17 (47%) AML patients for RHAMM/HMMR-derived, in 7/10 (70%) for PRAME-derived, and in 6/10 (60%) for newly characterized G250/CA9-G2-derived peptides. Furthermore, we could demonstrate specific lysis of T2 cells presenting these epitope peptides. *Conclusion.* The expression of the TAAs RHAMM/HMMR, PRAME and G250/CA9 can induce strong anti-leukemic immune responses possibly enabling the control of MRD in AML patients. Thus, these TAAs represent interesting targets for polyvalent immunotherapeutic approaches.

0110

PROGNOSIS OF ACUTE MYELOID LEUKEMIA PATIENTS < 60 YEARS WITH TRISOMY 8 AS A SOLE ABERRATION: POOLED DATA ANALYSIS OF THE GERMAN ACUTE MYELOID LEUKEMIA INTERGROUP

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Background. Trisomy 8 (+8) occurs in about 8-13% of patients with acute myeloid leukemia (AML). However, so far the prognostic impact and best consolidation strategy of this recurrent aberration is unclear. Thus, additional prognostic factors are needed to further classify those patients and to deliver appropriate treatment. *Methods.* Pooled data analysis was performed on 198 adult patients (median age 49 (17-60) years) with +8 treated between 1993 and 2002 in eight prospective German AML treatment trials. Patients with t(8;21), inv(16) or abn11q23 and an additional +8 were not included in the study. Clinical, diagnostic and laboratory data were reviewed for consistency and completeness before analysis by a central coordination center. *Results.* Ninety-two (46%) patients had +8 as a sole aberration, 39 (20%) had one additional secondary aberration and 67 (34%) had +8 within complex karyotypes with at least three independent abnormalities. Patients with +8 as a sole aberration had a 3-year overall (OS) and relapse-free survival (RFS) of 27% (95%-CI 18%-36%) and 31% (95%-CI 18%-43%), respectively. Multivariate analysis including standard clinical and laboratory data, as well as percentage of +8 positive metaphases and FLT3 status revealed extramedullary disease at diagnosis as a significant prognostic variable for worse survival (HR 2.56 (95%-CI 1.38-4.75); p=.003), whereas post-remission therapy (i.e. high-dose cytarabine vs. autologous vs. allogeneic stem cell transplantation) did not influence survival. *Conclusion.* AML patients with +8 as a sole aberration can be stratified by extramedullary disease at diagnosis into two prognostic groups. However, alternative treatment approaches are needed to achieve more durable remissions in this AML entity in the future.

0111

PROGNOSTIC IMPACT OF THE NPM1/FLT3 ITD MUTATION STATUS IN ELDERLY PATIENTS >60 YEARS OF AGE WITH NORMAL KARYOTYPE AML: RESULTS OF AMLSG TREATMENT TRIAL AML HD98B

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Background. NPM1 mutations have been identified in approximately 50% of patients with normal karyotype acute myeloid leukemia (AML) and thus represent the most frequent genetic alteration in this subset of patients. In three recent studies mainly involving younger adults, statistical analysis revealed a significant interaction between NPM1 mutations and FLT3 internal tandem duplications (ITD). Only patients with NPM1 mutation in the absence of FLT3 ITD had a significantly better relapse free (RFS) and overall survival (OS). *Aims.* To evaluate the prognostic impact of the combined NPM1/FLT3 ITD mutation status on response to therapy, RFS and OS in elderly AML (> 60 years) with normal karyotype. *Methods.* So far, sequencing of NPM1 exon 12 mutations was performed in diagnostic samples from 84 patients entered into the AML HD98B treatment trial of the AML Study Group (AMLSG). Treatment included randomized induction therapy consisting of 2 courses of ICE (idarubicin, cytarabine, etoposide) with or without all-trans retinoic acid (ATRA) followed by first consolidation therapy with a course of HAM (cytarabine, mitoxantrone) with or without ATRA. For further postremission therapy, patients were randomized to one cycle intensive second consolidation therapy (idarubicin, etoposide) or 12 monthly courses of outpatient maintenance therapy (idarubicin and etoposide per os). *Results.* NPM1 mutations were identified in 43% of the leukemias. In analogy to the studies performed in younger AML, statistical analysis revealed a significant interaction of NPM1 and FLT3 ITD mutations. Only the NPM1+ /FLT3 ITD- genotype predicted for high response to induction therapy and better survival probabilities. Complete remission rate (CR) of this subgroup was significantly better than that in the other three subgroups (NPM1-/FLT3 ITD-; NPM1+/FLT3 ITD+; NPM1-/FLT3 ITD+) (66% versus 36%, $p<0.001$). Treatment failure in the three latter groups was due to a higher degree of refractory leukemias (55% vs. 20.5%). The higher response to therapy translated into significant better RFS ($p=0.001$) and OS ($p=0.01$) probabilities in the NPM1+/FLT3 ITD- compared to the three other genotypes. *Conclusions.* The NPM1+/FLT3 ITD- genotype defines a distinct subset of elderly AML patients with a favorable outcome. These data are most relevant when aiming at selecting those elderly patients who may benefit from intensive therapy and those who will not.

0112

PROGNOSTIC DETERMINANTS IN ADULT AML PATIENTS WITH INTERMEDIATE RISK KARYOTYPE

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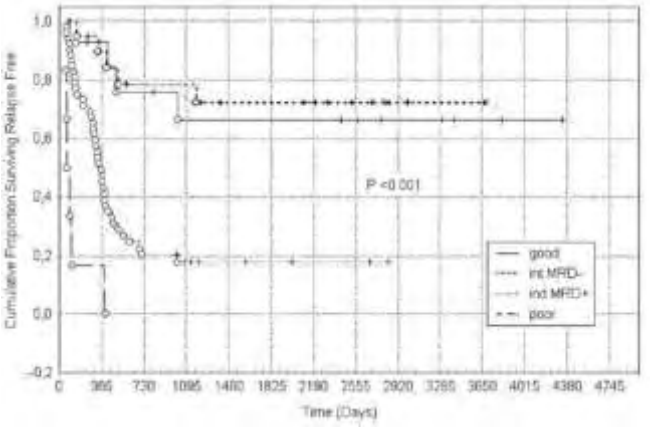
Background. According to the prognostic classifications of karyotypic abnormalities of AML (MRC and SWOG), the intermediate group includes patients either lacking good and poor karyotype or with normal karyotype. Therefore, it represents, by definition, a miscellaneous group for which the evaluation of the better treatment strategy is difficult due to its heterogeneity. Moreover, patients belonging to this intermediate group account for the large majority of AML cases enrolled into clinical trials. *Aims.* The aim of our study was to analyze the factors specifically affecting the outcome of patients bearing intermediate risk karyotypic abnormalities in a group of 94 AML cases entered into the EORTC/GIMEMA protocols AML10/AML12 (age <61yrs) or AML13/AML15 (age >61 yrs), consisting in intensive induction and consolidation cycles. *Methods.* The clinico-biological variables evaluated in our model included age, FAB, WBC count, MDR1 phenotype, FLT3 mutations and level of post-consolidation bone marrow residual leukemic cells (BMRCL) assessed by multiparametric flow-cytometry (MPFC). By applying the maximally selected log-rank statistics, the threshold discriminating MRD negative from positive cases was set at 3.5×10^{-4} BMRLC,. a level that allowed the identification of distinct subgroups of MRD- and MRD+ patients, both at post-Ind and post-Cons time-points. *Results.* Patients with $<3.5\times10^{-4}$ BMRLC at the end of consolidation therapy were considered MRD- and showed a better outcome, patients whose level of MRD were $>3.5\times10^{-4}$ at the end of consolidation were considered MRD+ and showed a poor prognosis. Using the MRC classification, 14/94 patients (15%) had a good-risk cytogenetics, 74/94 (79%) an intermediate-risk and 6/94 (6%) a poor-risk. When we restricted the analysis to cases with intermediate-risk karyotype we found that: 1) patients in the MRD- and MRD+ group differed significantly in terms of relapse free survival (RFS), overall survival (OS) and relapse rate ($p<0.001$, 0.006 and <0.001, respectively); 2) MRD- patients had an outcome slightly better than those bearing good risk karyotype; 3) MRD+ patients showed a dismal outcome comparable to poor-risk cytogenetic patients. *Conclusions.* These results suggest that the inclusion of MPFC assessment of MRD in patients with intermediate risk karyotype may be particularly useful in discriminating subgroups with different outcomes, in a group of AML where karyotype does not represent a clear prognosticator, allowing clinicians to design risk-based therapeutic programs.



0113

ROLE OF DNA METHYLTRASFERASES (DNMTS) AND POLYCOMB GROUP OF PROTEINS IN LEUKEMIA

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PML-RAR induces a block of hematopoietic differentiation and acute promyelocytic leukemia.[1] This block is based on its capacity to inactivate target genes by recruiting histone deacetylase (HDAC) and DNA methyltransferase activities.[2] Here we report that MBD1, a member of a conserved family of proteins able to bind methylated DNA, is required for silencing the PML-RAR target promoter RARb2.[3] After PML-RAR-induced promoter hypermethylation, MBD1 is recruited to and remains associated with the silenced RARb2 promoter. Mutations in the MBD domain and transrepression domain (TRD) of MBD1 restore RARb2 transcriptional activity and prevent PML-RAR-induced hematopoietic differentiation block. We provide evidence that HDAC3 is a common interactor for both PML-RAR and MBD1. APL cells knocked down for HDAC3 are impaired in PML-RAR mediated gene silencing. Our findings demonstrate (i) a targeting mechanism for MBD recruitment by an oncogenic transcription factor, (ii) a direct role of MBD1 and HDAC3 in promoter silencing and in leukemia progression, and (iii) a time-dependent spreading of MBD1 occupancy outside of the promoter region. Together these results identify MBD1 as a critical mediator of PML-RAR -induced gene silencing subsequent to promoter hypermethylation.

Further characterization of the PML-RARa-co-repressor complex, which establishes and allows spreading of the silenced state via Polycomb complex, will be additionally presented, together with the implication of crosstalk among the different epigenetic layers to the molecular pathology of leukemia.[4]

Reference

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0114

TRISOMY 8 AS SOLE ANOMALY OR WITH OTHER CLONAL ABERRATIONS IN ACUTE MYELOID LEUKEMIA: IMPACT OF CLINICAL PRESENTATION AND TREATMENT OUTCOME

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Background. Trisomy 8 is the most frequent numerical aberration in acute myeloid leukemia (AML). It occurs either as the sole anomaly or together with other clonal chromosome aberrations. Only few data are available regarding their prognostic significance. *Aims.* In order to investigate whether accompanying chromosome anomalies influence the clinical outcome in patients with trisomy 8, we assess clinical and biological characteristics, and response to therapy, of an unselected group of patients with previously untreated AML, presenting with trisomy 8 either alone or with other clonal aberrations. *Methods.* One hundred and fifty-four cases (median age: 65 years) were diagnosed in our institution between 1981 and 2005 including 47 patients (31%) with trisomy 8 as the sole aberration, 107 patients (69%) with trisomy 8 associated with other cytogenetic abnormalities (13 with favorable risk, 54 with intermediate risk, and 40 with unfavorable risk cytogenetics). *Results.* Twenty-eight patients only received symptomatic therapy or died before any chemotherapy could be given. All other patients received induction treatment according to different protocols used during the period of study. Overall complete remission (CR) proportion was 48% (95% confidence interval (CI): 40 - 56%). Sixty-six patients achieved CR after one course of chemotherapy and 8 patients after salvage therapy. Median disease-free survival (DFS) of the entire cohort was 7.8 months (95% CI: 6.5-9.9 months) and median overall survival (OS) was 8.3 months (95% CI: 5.2 ' 9.8 months). In multivariate analysis, age more than 60 years and trisomy 8 associated with unfavorable chromosomal aberrations were of poor prognostic value for CR achievement. Age more than 60 years and antecedents of dysmyelopoiesis were of poor prognostic value for DFS and OS. Patients with trisomy 8 alone did not show a significant difference in terms of outcome as compared with those in whom trisomy 8 was associated to intermediate risk chromosomal aberrations. Patients with trisomy 8 in addition to favorable chromosome aberrations maintained a good clinical outcome, while those with trisomy 8 in addition to unfavorable karyotypes showed the worst prognosis. *Conclusions.* Trisomy 8 as a whole has poor survival, which is largely attribuable to worsened outcomes among patients whose trisomy 8 was associated with unfavorable cytogenetic abnormalities. A particular poor outcome was observed in patients presenting trisomy 8 with antecedents of myelodysplasia.

0115

ANALYSIS OF 1458 ACUTE MYELOID LEUKEMIA FROM THE ALERT PROJECT (ACUTE LEUKEMIA CLINICAL REGISTER) IN THE CZECH REPUBLIC IN 1996-2006

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Project ALERT was initiated 9 years ago as population registry for acute leukemias, based on the cooperation of large haematological centres in the Czech Republic. Although the primary aims were rather clinical than epidemiological, the growing database tends to become representative for the Czech population, at least in the category of intensively cured AML patients. The database is improved to provide patients parametric collection of data gathered in accord with the therapeutic protocols and is full-scale record of prognostic markers including cytogenetic maps. The now presented and analysed cohort consists of 1458 AML patients registered in ALERT from January 1996 to December 2005. Median age of the patients was 58 years (32-74 year 10-90% kvantil limit). 636 (44%) patients were ≥55y and 822 (56%) older than 55y. The patients were observed with median 2y (0-5.5 y 5-95% kvantil limit). 90.4% of younger group pats were treated by intensive induction (CR rate 76%, median OS 17 months, DFS 22m and OS of the patients that achieved CR was 47.5 m). No differences were found between different induction intensity regimens. In the older group out of 56% of patients that received induction treatment 53% pats achieved CR with median OS 9m (DFS 11.2 m and OS of the patients achieved CR was 20 m). Out of 435 younger pats 18.5% were treated with standard dose consolidation, 38% with intermediate or HDCH and 39% with SCT (31% Auto SCT, 20% MUD SCT and 49% sibling donor SCT). In the older group out of 254 patients treated with consolidation treatment 37% were treated with standard dose consolidation CH, 43% with ID or HDCH and 10% only with SCT. The differences for OS and DFS with different consolidation treatment regimens will be given. The analysis of prognostic significance of age confirmed age over 55 as poor prognostic factor. The cytogenetic results of 758 intensively treated patients were studied. These include 103 PML patients. More than 80% of these patients live in molecular CR. The stratification of cytogenetic data for very good prognosis (PML), good prognosis, standard prognosis and poor prognosis confirmed their prognostic significance for OS, but surprisingly there were not found prognostic differences for DFS between standard and good prognostic group. Very important result revealed the analysis of centers showing comparable therapeutic results in all centers treating AML in the Czech Republic, even if they do not use the same therapeutic protocols for the treatment of AML. More detailed data will be given in the presentation.

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0116

CLORETAZINE (VNP40101M) HAS SIGNIFICANT ACTIVITY AS INDUCTION THERAPY FOR ELDERLY PATIENTS WITH ACUTE MYELOID LEUKEMIA OR ADVANCED MYELODYSPLASTIC SYNDROME

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Background. The incidence of AML increases with age with a median age of 68 years at diagnosis. Elderly pts with AML are more likely to have adverse prognostic factors related to the biology of the disease (adverse cytogenetics, history of MDS or prior exposure to cytotoxic agents, increased expression of multi-drug resistance) and medical comorbidities. Little progress has been made in improving outcomes in older pts. Despite variations on the 7+3 regimen, most elderly pts do not receive cytotoxic chemotherapy; pts who are treated with current regimens have lower response rates and overall survival, as well as poorer tolerance of side effects, compared to younger pts. New agents are required to increase complete remission (CR) rate and duration with improved safety in this population. Cloretazine, is a novel alkylating agent that has shown significant anti-leukemia activity *in vitro* and *in vivo* models. *Aims.* A multi-center Phase II study was conducted to investigate activity and safety of Cloretazine, in pts ≥60 years old with newly diagnosed AML or high risk MDS. *Methods.* Cloretazine, was administered at 600 mg/m^2 as a single 30-60 min. IV infusion. Second induction was allowed for patients who showed improvement. Patients who achieved CR or CRp could receive a consolidation course of 400 mg/m^2.

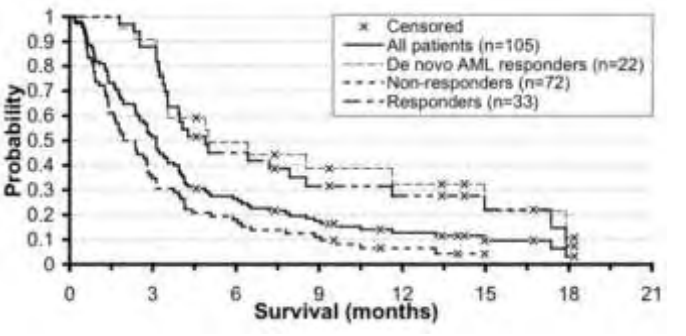

Figure 1.% Probability of Overall Survival.

Results. 105 pts were treated (median age 72, range 60-84), of whom 45 (43%) had *de novo* AML, 45 (43%) had secondary AML and 15 (14%) had high risk MDS. Twenty-eight pts achieved a CR and 5 pts a CRp for

an overall response rate of 31%. Response in 45 *de novo* AML, 45 secondary AML and high risk MDS was 49%, 11% and 40%, respectively. Response by cytogenetics was 42% in 57 intermediate pts and 22% in 41 unfavorable pts. The CR rates achieved with Cloretazine, are consistent despite increasing age and declining performance status. Severe drug-related non-hematologic toxicity was rare. Nineteen (18%) pts died within 30 days of receiving Cloretazine,. As demonstrated in the figure below, the 1-year overall survival for all treated pts (N=105) was 12%, and 28% for pts with CR (N=33). Patients with *de novo* AML who achieved CR had a median survival of 5 months and a 1-year survival of 32% (N=22). Conclusion: Cloretazine, is well tolerated and has significant activity in an elderly patient population with AML or MDS. The encouraging activity in patients with *de novo* AML warrants further evaluation.

0117

DOSING OF TROXATYL (TROXACITABINE, SGX-145) BASED ON RENAL FUNCTION

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Background. Troxatyl™ (troxacitabine, SGX-145) is a novel L-configuration nucleoside analog with unique mechanistic and cytotoxic properties. Troxacitabine is clinically active in patients with relapsed or refractory acute myelogenous leukemia (AML), including those who have failed bone marrow transplantation. Troxacitabine has also demonstrated clinical activity against chronic myeloid leukemia, myelodysplastic syndromes, renal cell carcinoma, and pancreatic cancer. An international (European and North American) multi-center Phase 2/3 clinical trial is currently underway to evaluate the safety and efficacy of troxacitabine continuous IV infusion treatment in second salvage AML (SPD758-216; www.clinicaltrials.gov). The major route of elimination of troxacitabine is renal excretion as unchanged drug (~70%) and there is no detectable protein binding. These results suggest that renal function may play a significant role in determining the troxacitabine blood concentration in an individual patient. *Aims.* The aims of this study were to determine the influence of renal function on troxacitabine steady-state plasma concentrations (Css), and to determine if a correlation exists between troxacitabine Css and clinical response. *Methods.* Pharmacokinetic and toxicity data from multiple AML clinical trials (enrolling >200 patients) are being analyzed to (1) Determine the relationship between troxacitabine Css values and estimated creatinine clearance; (2) Identify the minimum troxacitabine Css required to achieve a clinical response (CR or CRp); (3) Define an upper limit of troxacitabine Css for adverse risk; and (4) Develop a dosing nomogram or equation to prospectively adjust troxacitabine dosing, based on patient renal function, which will allow avoidance of excessive toxicity while still achieving therapeutic blood levels. *Results.* Initial results indicate that in order to induce remission in relapsed or refractory AML patients, troxacitabine Css values must ≥ 80 ng/mL. High troxacitabine Css values may correlate with increased toxicity, although an upper limit for adverse risk has not yet been defined. For patients with normal to mildly impaired renal function (creatinine clearance > 45 but < 125 mL/min), a Calvert style formula, based on estimated creatinine clearance, has been developed to define the minimum dose required to achieve the target troxacitabine Css of > 80 ng/mL. Both linear and non-linear nomogram models are being developed to adjust troxacitabine dosage for patients with moderate impairment of renal function (creatinine clearance < 45 mL/min) or for patients with higher glomerular filtration rates (creatinine clearance > 125 mL/min). *Summary*. A firm relationship exists between renal function and troxacitabine Css values, and between troxacitabine Css values and clinical response in patients with relapsed or refractory AML. These results indicate that developing a dosing strategy, based on patient renal function, may be warranted to obtain optimal troxacitabine Css values with minimal toxicity.

0118

CYTOGENETIC RESPONSES IN OLDER PATIENTS WITH AML AND COMPLEX KARYOTYPE TREATED WITH LOW- DOSE 5-AZA-2-DEOXYCYTIDINE (DECITABINE)

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Background. The demethylating agent Decitabine (DAC) induces hematologic and cytogenetic remissions in older patients (pts) with MDS and AML (Rüter *et al.* Int J Hematol. 2004; 80: 128-35). Cytogenetic normalization was more frequently seen in pts with poor-risk cytogenetics (mostly complex karyotype) compared to intermediate-risk (Lübbert *et al.* Br J Haematol 2001; 114: 349). *Aim.*Prospective analysis of induction of cytogenetic and hematologic responses in AML pts aged > 60 (ineligible for induction chemotherapy) treated with the *MDS low-dose* DAC schedule within a phase II study. *Methods.* We systematically evaluated sequential cytogenetics in 39 consecutive pts (median age 73 years, range 63-86) treated at two study centers. Pts aged > 60 years diagnosed with AML (according to FAB, >30% blasts) were treated with DAC (15mg/m^2 in a total of 9 doses over 72 hours) every 6 weeks for up to 4 courses, with all-trans retinoic acid (ATRA, 45mg/m^2/day for 28 days) given during course 2 in DAC-sensitive pts. For pts having successfully completed all 4 courses a maintenance treatment with 20 mg/m^2 DAC i.v. over 1 hour on 3 days (total dose 60 mg/m^2, outpatient administration) every 8 weeks was offered. Cytogenetics were performed before course 1-3 and at the end of course 4. *Results.* Of 35/39 successfully karyotyped pts 23 had chromosomal abnormalities prior to treatment. Cytogenetic subgroups were: good risk: 0; intermediate risk: 21 pts (12 with normal karyotype); poor risk: 14 pts. Pts received a median of 2 DAC courses (range 1-4) and 10 pts a median of 2 maintenance courses (range, 1-9). 21/35 pts received ≥ 2 courses of low- dose DAC (17 also ATRA) and were evaluable for cytogenetic remissions. 13 of the 21 pts (62%) had chromosomal abnormalities at diagnosis (9 with complex karyotype). 3/13 pts had a complete cytogenetic normalization, all with a complex karyotype at time of diagnosis (aberrations of chromosome 5 in 3, of chromosome 7 in 2). The cytogenetic response occurred after a median of 2 courses. Overall hematologic response in the 39 pts by intent-to-treat (ITT) was 43% (17/39) with 6 CR (15%), 4 PR (10%) and 7 antileukemic effect (ALE) (18%). *Conclusions.* Low-dose DAC is active, by ITT, in inducing hematologic response in 43% and cytogenetic normalization in 13% of older AML pts (according to FAB), all 3 with complex karyotype. In MDS pts cytogenetic normalization (after a median of 4 courses) had been seen more frequently (31%). AML pts received a median of 2 courses which could explain at least in part the lower cytogenetic response rate.

0119

SINGLE POINT MUTATIONS IN THE JUXTAMEMBRANE DOMAIN OF FLT3 ARE RARELY FOUND IN AML PATIENTS AND DEFINE THE THIRD CLASS OF ACTIVATING MUTATIONS

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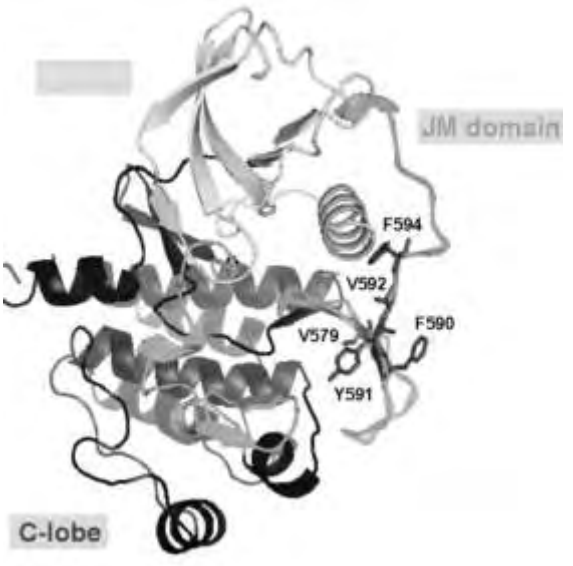
Background. In acute myeloid leukemia (AML) FMS-like tyrosine kinase-3 (FLT3) is mutated in about 30% of the patients. Until now, two clusters of activating FLT3 mutations are known: FLT3-internal tandem duplications (FLT3-ITD) in the juxtamembrane (JM) domain in 20-25% and FLT3-point mutations in the tyrosine-kinase domain (FLT3-TKD) in 7-10% of patients, respectively. *Aims.* By sequencing the complete cDNA of FLT3 in the AML cell lines MM6 and MM1 we have previously found a new activating point mutation in the JM domain of FLT3: V592A (Spiekermann, Blood 2003). Therefore we established a LightCycler screening assay to analyze AML patient samples for point mutations in the JM domain of FLT3. *Methods.* By using a LightCycler screening assay we analyzed 785 unselected AML samples for mutations in the region surrounding V592 of FLT3. The mutated FLT3 receptors were stably expressed in Ba/F3 cells and characterized by proliferation and apoptosis assays. FLT3 activation status and downstream signaling pathways were analyzed. Structural analysis was performed with PyMOL software. *Results.* The LightCycler screening of AML samples identified two point mutations in the JM domain of FLT3 (FLT3-JM-PM): Y591C and F594L. Stirewalt *et al.* also recently investigated the JM domain of FLT3 and could detect three point mutations: V579A, F590GY591D and V592A (Stirewalt, Br J Hematol 2004). Stable expression of four FLT3-JM-PM in Ba/F3 cells led to factor-independent growth, hyperresponsiveness to FLT3-ligand and resistance to apoptotic cell death. FLT3-JM-PM receptors were constitutively autophosphorylated and showed a higher constitutive dimerization rate compared to FLT3-WT receptors. Analysis of downstream signaling pathways could show activation of STAT5 and upregulation of Bcl-x(L) by all FLT3-JM-PM. The selective FLT3-inhibitor PKC412 abrogated the factor-independent growth of FLT3-JM-PM and totally inhibited STAT5 phosphorylation. Compared to FLT3-ITD and FLT3-TKD expressing Ba/F3 cells, the FLT3-JM-PM

showed a weaker gain-of-function phenotype in terms of proliferation, anti-apoptosis, activation of FLT3 and the downstream target STAT5. As the FLT3-JM-PM cluster at a core interaction site of the JM domain with the remainder of the molecule we hypothesized that the oncogenic potential results from the perturbation of the autoinhibitory mechanism. Mapping of the FLT3-JM-PM on the crystal structure of FLT3 (Figure) could show that these mutations probably reduce the stability of the autoinhibitory JM domain. In comparison to FLT3-ITD mutations, that increase the length of JM, the FLT3-JM-PM induce a considerably weaker perturbation and this might provide a structural basis for the weaker (FLT3-JM-PM) versus stronger (FLT3-ITD) transforming capacity of these mutations. *Summary*. A third class of activating FLT3 mutations exists in 2% of AML patients, point mutations in the structurally important JM domain (FLT3-JM-PM). Patients carrying FLT3-JM-PM might benefit from the treatment with selective FLT3 inhibitors. FLT3-JM-PM provide a remarkable example how mutations disturbing the autoinhibitory JM domain of class III RTK can contribute to the pathogenesis of cancer.



0120

ORIGINAL DATA BASED META-ANALYSIS ON PATIENTS >60 YEARS OF AGE WITH CORE BINDING FACTOR ACUTE MYELOID LEUKEMIA RESULTS OF THE GERMAN AML-INTERGROUP

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Background. Acute myeloid leukemias exhibiting t(8;21) or inv(16) karyotypes are referred to as core binding factor (CBF) acute myeloid leukemia (AML). In younger patients with CBF-AML relapse free survival (RFS) was markedly improved following dose-intensified cytarabine for consolidation therapy. However, in older patients (>60 years) the clinical course is characterized by significantly inferior outcome and the value of dose-intensified cytarabine remains unclear. *Aims.* To review the clinical course of CBF-AML in a large cohort of elderly patients and to define the relative value of different treatment strategies. *Methods.* We performed a meta-analysis on 65 patients with t(8;21) and on 51 patients with inv(16) from 4 German leukemia study groups. The patients were treated in 5 different prospective multicenter treatment trials between 1995 and 2004 (DSIL n=36, AMLSG n=26, AMLCG n=34, OSHO n=20). Induction therapy consisted of standard dose cytarabine (ARAC) combined with etoposide/idarubicine or mitoxantrone, or dose-intensified cytarabine in combination with idarubicin or mitoxantrone; postremission therapy consisted of intensive chemotherapy followed by maintenance therapy in two trials. *Results.* The median age was 66 years (range 61-85) and median follow up time was 55 months. Response to induction therapy for t(8;21) and inv(16) was as follows: complete remission (CR) 72.5% and 86%, refractory disease (RD) 12% and 10%, early/hypoplastic deaths 15.5% and 4%, respectively. RFS and overall survival (OS) after 4 years were for t(8;21)-AML 20% (95%-CI 11-38%) and 20% (95%-CI 11-35%) and for inv(16)-AML 35% (95%-CI 23-54%) and 33% (95%-CI 22-51%), respectively. To evaluate the impact of dosage of cytarabine on outcome patients were categorized into the HiDAC-group if they received at least one cycle of high-dose cytarabine with a cumulative dosage of ≥6g/m^2 (n=35) or otherwise into the STANDARD-dose cytarabine group (n=34). RFS was significantly (p=0.002) better in the HiDAC-group (44%, 95%-CI 30-65%) compared to the STANDARD-group (16%, 95%-CI 7-35%). *Conclusion.* Elderly patients with CBF-AML seem to benefit from dose-intensification of cytarabine above or equal to 6g/m^2 in at least one treatment cycle.

0121

THE KINETICS OF REDUCTION OF MINIMAL RESIDUAL DISEASE IMPACTS ON DURATION OF RESPONSE AND SURVIVAL OF PATIENTS WITH ACUTE MYELOID LEUKEMIA

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Background. We assessed by multiparametric flow-cytometry the levels of minimal residual disease (MRD) in 100 adult patients with acute myelogenous leukemia (AML) achieving complete remission after intensive chemotherapy. *Aims.* The aim of the present study was to determine the optimal threshold level, in term of residual leukemic cells (RLC), and the time-pont of choice, i.e. post-induction (post-Ind) or post-consolidation (post-Cons), capable to better predict outcome of AML patients. *Methods.* By applying the maximally selected log-rank statistics, the threshold discriminating MRD negative from positive cases was set at 3.5×10^{-4}RLC, a level that allowed the identification of distinct subgroups of MRD' and MRD+patients, both at post-Ind and post-Cons time-points. *Results.* Post-Cons MRD' patients had a superior outcome in terms of relapse rate, OS and RFS (p<0.001, for all comparisons), regardless of the MRD status after induction. In particular, patients entering MRD negativity only after consolidation showed the same outcome as those achieving early negativity after induction. Multivariate analysis including karyotype, age, MDR1 phenotype, post induction and post consolidation MRD levels indicated that the post-consolidation MRD status was an independent factor affecting relapse rate, OS and RFS (p<0.001, for all comparisons). *Conclusions.* 1) the threshold of 3.5×10^{-4} is valid in discriminating risk categories in adult AML; 2) MRD assessment at post-consolidation time-point is critical to predict disease outcome.

0122

CLOFARABINE AS FIRST-LINE TREATMENT OF ELDERLY (=65 YRS) AML PATIENTS WITH AN UNFAVOURABLE CYTOGENETIC PROFILE WHO ARE UNSUITABLE FOR STANDARD TREATMENT

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Background. BIOV-121 is a phase II non-randomised trial of clofarabine, a next generation purine nucleoside analogue, in older patients (≥65 yrs) with previously untreated acute myeloid leukemia (AML) who are unsuitable for standard (3+7) chemotherapy. *Aims.* The primary endpoint of study BIOV-121 is to determine the overall response rate (ORR) of clofarabine in this elderly (≥65 yrs) AML population who are unsuitable for standard treatment. ORR is defined as the sum of the number of patients who achieve a complere response (CR), a complete response with incomplete peripheral count recovery (CRi) and a partial response (PR) according to the international working guidelines. Secondary endpoints include duration of remission, time to progression, and the safety and tolerability of clofarabine in this patient population. *Methods.* 66 patients aged ≥65 yrs with untreated AML defined by the WHO classification were enrolled in BIOV-121. All patients were considered unsuitable for standard treatment based primarily on age (≥65 yrs) and/or performance status. All patients received clofarabine 30mg/m^2 for 5 days repeated every 28 days (1 course). A preliminary analysis was conducted for patients with an unfavourable cytogenetic profile (16/63), for whom data was available, and for patients >70 years of age (36/66). Unfavourable cytogenetic profile was defined as the presence of a com-

plex karyotype, monosomies of chromosome 5 (del[5q]/5q-), or 3q abnormalities as reported by Grimwade et al (U.K. MRC AML 10 trial). *Results.* Of 63 patients, 25% (16/63) had an unfavourable cytogenetic profile. The median number of clofarabine courses administered was one. With a median age of 66 years the overall response rate (ORR) in this unfavourable cytogenetic patient group was 50% (8/16) with a 44% complete response (CR/CRi) rate. 55% (36/66) of patients were aged >70 years. The ORR and CR rate was 56% and 44% respectively in patients aged > 70 years.The safety profile of clofarabine was acceptable and manageable in this elderly AML population considered unsuitable for standard treatment. *Summary/Conclusions.* Clofarabine demonstrates efficacy as first-line treatment of elderly (≥65 yrs) AML patients with an unfavourable cytogenetic profile, considered unsuitable for standard (3+7) chemotherapy.

0123

ABCB1 (PGP) AND ABCG2 (BCRP) PROTEINS ARE INDEPENDENT PROGNOSTIC FACTORS FOR COMPLETE REMISSION AND RELAPSE RISK, RESPECTIVELY, IN NORMAL KARYOTYPE ADULT *DE NOVO* ACUTE MYELOID LEUKEMIA

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Background. Multidrug resistance (MDR) is a major cause of failure in acute myeloid leukemia (AML) therapy. P-glycoprotein (PGP) has demonstrated a high prognostic power, as a negative correlation between PGP over-expression, remission rate and survival has been observed in different studies on AML. Recently a new ATP-binding cassette protein, the breast cancer resistance protein (BCRP), has been identified. In cell line systems BCRP confers resistance to many different compounds and plays an important role in affecting drug disposition, while its effective role *in vivo* is much less defined. *Aims.* We have compared the expression of BCRP and PGP in 73 consecutive cases of normal cytogenetic AML, in the attempt to identify another prognostic factor potentially useful to design a risk-adapted therapy. *Methods.* Seventy-three patients with a diagnosis of *de novo* AML with normal karyotype were included in our study. Median age was 53 years (range: 15-76) and 35 patients (48%) were older than 55 years. Patients were homogenously treated, with an induction regimen containing fludarabine, cytarabine and idarubicin, and a consolidation course with high-dose cytarabine and idarubicin. Complete remission (CR) was defined after two courses of therapy, according to the published criteria. Twenty-five patients considered at high risk of relapse and with an identical donor underwent allogeneic stem cell transplantation (SCT). *Results.* BCRP protein was over-expressed in 24/73 (33%) patients. BCRP positive cases showed a higher expression of CD56 antigen (11/24, 46%) compared to BCRP negative patients (10/47, 21%) (p=0.03). Similarly, PGP was more frequently over-expressed in patients with a concomitant expression of BCRP (13/24, 54%) than in BCRP-negative cases (11/49, 22%) (p=0.006). CR was obtained in 55/73 (75%) patients. Only advanced age, high level of PGP and CD34 expression affected remission rate in the univariate analysis. The first two factors retained their statistical significance also in the multivariate analysis, while CD34-positivity showed a strong trend toward significance (p=0.06). On the contrary, BCRP expression was not associated with CR obtainment. However a significantly higher probability of relapse was observed in patients with high BCRP expression: 14 out of 18 (78%) BCRP-positive patients relapsed, compared to only 14/37 (38%) in the BCRP-negative group (p=0.005). No other parameters were associated with an increased relapse risk. BCRP over-expression affected also disease free survival (8 vs 27 months, p=0.027). PGP expression, which is one of the strongest predictors of remission, did not influenced DFS. Finally, a shorter survival was associated with response to induction therapy (CR or not, p=0.02), CD56 positivity (p=0.03) and by the expression of at least one MDR-associated protein (p=0.05). *Summary/Conclusions.* BCRP was over-expressed in a significant percentage of AML patients with normal karyotype. BCRP over-expression did not influence achievement of remission, as PGP did, but significantly affected CR duration. BCRP-positive patients displayed a significantly higher relapse rate. BCRP may be therefore regarded as an easy evaluable prognostic factor in AML with normal karyotype, and should help in the design of a risk-adapted post remission therapy.

Acute myeloid leukemia II

0124

IMMUNOPHENOTIPIC PATTERN OF HIGH RISK KARYOTYPE ACUTE MYELOID LEUKEMIA IS CHARACTERIZED BY EXPRESSION OF CD34 AND LACK OF MPO

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Background. Cytogenetic is nowadays the most remarkable prognostic factor in acute myeloid leukemia (AML), and immunophenotypic analysis may help to identify some of the most frequent cytogenetic abnormalities in this disease. *Aim.*To identify immunophenotypic patterns present in AML with adverse cytogenetics. *Methods.* Samples from a total number of 185 *de novo* AML patients (median age: 54 years, range 10-91; 56% male and 44% female) were immunophenotypically analyzed by multiparametric flow cytometry, using a large panel of 25 monoclonal antibodies. A cut-off point of 20% of the total blast cell population was used to define a marker as positive or negative. Chromosome analysis of bone marrow cells was performed at diagnosis using short-term (24-48 h) unstimulated cultures. At least 20 metaphases were analyzed. Definitions of cytogenetic clonality and karyotypic descriptions were in accordance with ISCN guidelines (1995). Cases with abnormalities in chromosome 3, 5, 7 or complex karyotype (3 or more abnormalities) were considered as adverse prognosis. *Results.* Among the AML cases classified as high risk karyotype, 32/59 (69%) showed a constant immunophenotypic pattern in blast cells characterized by the CD34 expression and lack of expression of cytoplasmatic myeloperoxidase. In contrast, this pattern was observed in 4/61 (9%) and 10/51 (22%) of good and intermediate cytogenetic risk group respectively (p<0.001). Moreover, when patients with chromosome 3 abnormalities were considered as an isolated group (n=9), this pattern was present in 89% of cases, while AML with -5/5q (n=10) and -7/7q (n=19) showed this immunophenotypic pattern in 50% and 53% of cases, respectively. By contrast, AML with good risk karyotypes (n=61) only showed this immunophenotipic pattern in 3/21 (14%) of AMLs with t(8;21), 1/21 (5%) of inv(16) and no cases with t(15;17). Therefore, once the CD34+/MPO- immunophenotypic pattern is identified, the relative risk of bearing a cytogenetic abnormality belonging to the adverse cytogenetic group in *de novo* AML patients is 3.2 times when compared to the immunophenotypic profiles. *Conclusions.*The immunophenotypic pattern characterized by the expression of CD34 and lack of expression of MPO (CD34+ MPO-) is associated with adverse prognosis karyotype. Additional cytogenetic studies (e.g. FISH) should be performed in cases with normal or unsuccessful cytogenetics analysis in order to identify possible overlooked abnormalities.

0125

ABNORMALITIES IN P53 AND P14ARF IN *DE NOVO* AML PREDICTS A VERY SHORT OVERALL SURVIVAL AND *IN VITRO* DRUG-RESISTANCE.

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The purpose of this study was to correlate the karyotype of myeloblast to long-term overall survival and *in vitro* cytoxicity of conventional antileukemic drugs in patients with *de novo* AML. Abnormalities in chromosome 17, which are associated with p53 mutations, and 9p, the locus that encodes among others p14ARF which binds and inactivates the HDM-2, which in turn targets p53 for degradation. Thus deletions in 9p21 resulting in inhibitory effects on p53 protein were focused on. *Methods.* Blast cells were isolated from 381 patients diagnosed with *de novo* AML during the last 20 years at our clinic. Chromosomal analysis was successful in 318 cases. All samples were tested for *in vitro* cytotoxicity for fludarabine, AMSA, mitoxantrome, vepeside, daunorubicine and Ara-C after 4days culture, using the ATP assay. *in vitro* cytotoxicity was correlated to chromosomal aberrations. In the 318 patients, five main groups were identified; cases with monosomy 7 or deletion 7q (n=32), complex karyotype (n=50), normal karyotype (n=114), abnormal chromosome 17 (n=20) or abnormal 9p (n=13). Complex karyotype and the chromosome 7 abnormalities are well known markers for poor prog-

nosis, long term outcome and *in vitro* drug resistance. The first three groups were compared to patient's samples with abnormal chromosome 17/9p. *Results.* Abnormalities of chromosome 17 indicate a significantly higher drug resistance for all drugs tested and a significant shorter overall survival compared to patients with normal and complex karyotype. A shorter overall survival and higher drug resistance was also noted when comparing abn.17 to patients with abnormal 7, but the differences was not significant. All patients with abnormalities on chromosome 17 died within eleven months after diagnosis. Patients with abnormal 9p had a shorter overall survival but did not differ in *in vitro* drug resistance compared to patients presented with normal karyotype. *Conclusions.* Abnormalities in chromosome 17 appears to be a strong marker for both *in vitro* drug resistance and adverse outcome, even when compared to other high risk karyotypes in AML. Patients with abnormalities in 9p, which affect p53 protein pathway and degradation, showed a shorter overall survival but less obvious drug resistance.

0126

LEUKEMIA IN UKRAINIAN CLEAN-UP WORKERS OF THE CHORNOBYL ACCIDENT: EPIDEMIOLOGIC AND HEMATOLOGIC ASPECTS

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Background. Leukemia holds a special place in the study of radiation-related cancer because bone marrow is one of the tissues most sensitive to the carcinogenic effect of ionizing radiation, radiogenic leukemia has the shortest latent period among radiation-induced cancers, and its appearance suggests that solid tumors may follow. U.S. National Cancer Institute (study team - A Bouville, M.Hatch, G Howe, N Luckyanov, I Masnyk, L Zablotska) and Research Center for Radiation Medicine from Ukraine (study team NG Babkina, E Bakhanova, EI Bomko, Yu Byelyayev, V Chumak, I Dyagil, NA Gudzenko, TF Lubarets) have initiated a study of radiogenic leukemia. *Aims.* The main objective is to test the hypothesis that exposure to radiation during cleanup operations following the Chornobyl accident led to an increase in leukemia among male cleanup workers from Ukraine. *Results.* A retrospective case-control study of ionizing radiation and leukemia was conducted in a cohort of 110,645 male Ukrainian *liquidators* involved in cleanup work following the accident at the Chornobyl nuclear power plant in northern Ukraine which occurred on April 26, 1986. The cohort includes 46% of clean-up workers in Ukraine. Information on all cases from 1986 to 2000 was collected in the hematological, pathological departments of local hospitals and registries of radiation exposed after Chornobyl in 5 target areas of Ukraine and Kyiv city including clinical records and blood smears, bone marrow slides, cytochemical and histological preparations, immunophenotype. Cases were evaluated by the international diagnostic review from 5 pathologists (2- USA, 2 - Ukraine, 1- United Kingdom). Annual case distribution showed a marked tendency to increase with age after the exposure. To assess the influence of ionizing radiation exposure analysis was performed of observed and expected (spontaneous) leukemia numbers at the cohort basing on the data on male population of Ukraine. The doses in cases obtained by RADRUE retrospective dosimetry were higher than in controls..Risk estimates were performed. *Summary.* Obtained data show the elevation of cases number and radiation risks in Chornobyl clean-up workers in Ukraine 8-14 years after the radiation exposure. Further follow-up of the cohort is performed.

0127

QUANTITATIVE ASSESMENT OF AML/ETO FUSION TRANSCRIPT AS USEFUL TOOL FOR MINIMAL RESIDUAL DISEASE DETECTION AND OUTCOME PREDICTION IN T(8;21) AMLS

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Background. The t(8;21) translocation derives from the fusion of AML1 on chromosome 21 and ETO on chromosome 8. It is associated with FAB subtype M2 acute myeloid leukemia (AML). In order to predict relapse, qualitative PCR has a limited value since a positive PCR can be observed for a long follow-up period , even during continuous complete remission (CCR). From preliminary studies, quantitative RT-PCR seems to be a good candidate to predict clinical outcome of patients presenting AML1-ETO rearrangement. *Aims.* To test the usefulness of quantitative RQ-PCR assay in detecting minimal residual disease and in predicting relapse in patients affected by t(8;21) AML. *Methods.* We analyzed in a retrospective manner 123 PB and BM samples from 33 patients affected by AML presenting AML1-ETO rearrangement by standard nested RT-PCR. 111 out of 123 BM samples were also analyzed by quantitative RQ-PCR technique following standard *Methods.* Samples were taken at diagnosis, after induction and consolidation therapy. *Results.* The median age of the patients was 34 years (range 6-60). 13 patients underwent conventional chemotherapy and 20 were treated with autologous or allogeneic bone marrow transplantation. WBC median value at diagnosis was 7905 (range 400-23300). 9 out of 21 patients who achieved and remained in CCR showed qualitative RT-PCR positivity after consolidation treatment, in two cases even after allogeneic and autologous transplantation showing a late clearance of the transcript. By contrast, 5 patients out of 12 achieved PCR negativity but subsequently relapsed. We compared the values obtained by RQ-PCR in the 21 patients who achieved complete remission to the 12 who relapsed during follow-up. At diagnosis quantitative analysis of AML-ETO fusion transcript showed a large variability (median value 71580, range 9632-801900). No difference was found between transcript amount at diagnosis and clinical outcome (p=0,6 by Mann-Whitney test). We did not observed any significant correlation between transcript amount and either WBC values (r= 0,14) or blast percentage (r= 0,23). Values obtained after induction treatment are not different in patients who reached CR as compared to those who relapsed (p=0,17). By contrast, after consolidation treatment, patients in CR showed a median transcript level of 14, compared to 393 AML-ETO copies in those who subsequently released.(p= 0,014). We also tried to identify a threshold level of transcript after consolidation to identify the CCR patients. We can observe that only two patients out of 21 in CCR reached a post consolidation value > 15 copy numbers. On the other hand only 4 out of 20 patients who obtained a post consolidation value below 15 copies subsequently relapsed. *Conclusions.* Quantitative RT-PCR assessment of AML/ETO fusion transcript amount in leukemic patients is a useful tool for detecting minimal residual disease. Our data demonstrated that the most significant value to predict final outcome is the one obtained after consolidation treatment. We can also identify 15 AML-ETO copies after consolidation as threshold level in order to predict patients' outcome.

0128

PROGNOSTIC SIGNIFICANCE OF BAALC EXPRESSION IN ACUTE MYELOID LEUKEMIA WITH NORMAL KARYOTYPE: FROM MICROARRAY TO RQPCR ASSAY

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Background. Clonal cytogenetic abnormalities are one of the most important factors predicting clinical outcome in Acute Myeloid Leukaemia (AML) and are used to guide risk-adapted treatment strategies. However, approximately 50% of *de novo* AML have normal karyotype and therefore lack informative chromosome markers. The identification of relevant genetic features as well as the discrimination between different subsets of patients within this group remain major challenges. BAALC high expression in pre-treatment blood samples (peripheral blast) was proposed to be an independent adverse prognosis factor for Overall Survival (OS) Event Free Survival (EFS) and Disease Free Survival (DFS). *Aims.* The goals of this study were to determine if expression of BAALC was a prognosis factor in AML by using a new specific quantitative PCR (RQ-PCR) assay and to compare these results with data obtained on microarrays. *Methods.* We developed a new specific RQ-PCR assay for quantification of BAALC transcripts in human cells. This assay is based on the plasmid technology which allows the precise calibration and normalisation of RQ-PCR results and is highly reproducible. The ratio of BAALC transcript to endogenous ABL transcript provides a normalised quantification of BAALC independent of the cell count and RT efficiency. We previously profiled on a 9000-cDNA microarray 61 adult AML samples with normal cytogenetic at diagnosis. Prognostic significance of BAALC and EVI1 expression levels was analysed using univariate Cox proportional hazards regression analyses. *Results.* Microarray data showed that BAALC expression values were associated with outcome (p=0.07). In addition, we could note that when combined with EVI1 expression values, results were even better. We thus could determine that: (i) EVI1 expression was significantly correlated with outcome in BAALC - group (p=0.03); (ii) BAALC+ and BAALC-/EVI1- patients correspond to a poor prognosis class and; (iii) BAALC-/EVI1+ patients to a good prognosis class (increased OS, p=0.0076) (Figure1). RQ-PCR assay analytical validation showed a reproducible sensitivity greater than 10-5 (less than 100 BAALC positive KG1a cells in

5.106 BAALC negative K562 cells or 50pg of KG1a RNA in 1µg of MV4-11 RNA). We determined BAALC levels in normal peripheral blood samples (n=26). The median normalised copy number (NCN) of BAALC was 2210 and ranged between 129 and 6427. Pathological values were assessed on a subset of AML samples (n=24) already used for microarray analysis. The median NCN was 1693, and ranged between 61 and 31850. The RQ-PCR results demonstrate a clear direct correlation (p=0.0057) between OS and expression of BAALC alone in normal karyotype AML patients (Figure 2). *Conclusions.* We confirmed high expression of BAALC as an adverse prognosis factor for normal karyotype AML patients, and identified a BAALC - / EVI1 + class with favourable outcome. The sensitivity and dynamic range of the BAALC RQ-PCR assay may contribute to treatment option decisions at diagnosis. Larger patient cohort and clinical trials will evaluate the impact of this new molecular tool on patient care. Further RQ-PCR studies will be performed to accurately assess the importance of EVI1 expression correlation with outcome in BAALC - group.

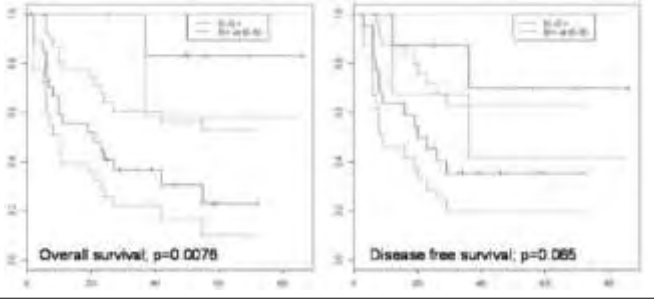

Figure 1. Microarray data analysis, OS and DFS.

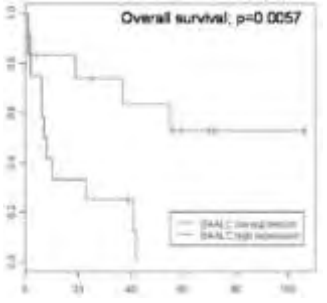

Figure 2. RQ-PCR data analysis.

0129

A MULTICENTER PROSPECTIVE RANDOMIZED STUDY OF LENOGRASTIM IN CONSOLIDATION CHEMOTHERAPY FOR ELDERLY PATIENTS WITH ACUTE MYELOID LEUKEMIA (JALSG GML200-S STUDY)

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Background. Although G-CSF administration after consolidation chemotherapy for elderly patients with acute myeloid leukemia (AML) is recommended in some clinical guidelines, effective use of G-CSF administration has been under investigation. *Aims.* We conducted a multicenter prospective randomized study to compare two different administration schedules of lenograstim after consolidation chemotherapy for elderly AML patients. The primary endpoint of this randomized study is to compare the duration of fever (above 38°). *Methods.* Patients with newly diagnosed untreated *de novo* AML older than 64 years of age who attained complete remission (CR) were eligible for this study. After consolidation chemotherapy with behenoyl cytarabine (200 mg/m^2, d1-5) and mitoxantrone (age<70 years; 7 mg/m^2, d1-3, age ≥70; 5 mg/m^2; d1-3), patients randomly assigned to receive lenograstim (5 µg/kg, 30 min i.v.) either after absolute neutrophil count (ANC) less than 1000/µL (Arm A: prophylactic administration) or after ANC less than 1000/µL with fever above 37.5° (Arm B: therapeutic administration) until neutrophil recovery. *Results.* Between August 2000 and March 2005, 110 evaluable patients were registered. 54 patients were in Arm A and 56 in Arm B, and the median age of both groups was 71 years old. There were no significant differences in patient characteristics. All patients received lenograstim in Arm A. Twenty-nine patients (51.8%) received lenograstim in Arm B, because 27 patients did not experience fever above 37.5°. The duration of fever was not significantly different between Arm A and Arm B (Mean±SD: 1.2±2.1 vs. 1.4±2.1 days, respectively, p=0.34). The duration of febrile neutropenia (ANC< 500/µL and temperature >37.5°) was slightly shorter in Arm A (Mean±SD: 1.2±2.4 as compared with 1.6±2.1 days in Arm B, p=0.078). In 65-69-year-old patients who received more intensive chemotherapy, the duration of febrile neutropenia was significantly shorter in Arm A (Mean±SD: 0.8±1.4, as compared with 2.0±2.5 in Arm B, p=0.032). The duration of infectious complications was slightly shorter in Arm A (p=0.06). The recovery of neutropenia was more rapid in Arm A compared to Arm B (ANC>500/µL: median 5.0 vs. 9.0 days, p<0.0001; ANC>1000/µL: median 8.5 vs. 14.5 days, p<0.0001). There was no difference of development of lenograstim-related severe toxicity between Arm A and Arm B. *Conclusions.* Prophylactic administration of lenograstim after consolidation chemotherapy obtained better clinical benefits, especially for those who received more intensive chemotherapy.

0130

INTERPRETATION OF INTERLEUKIN-2 RECEPTOR A POSITIVE CELLS DURING INDUCTION CHEMOTHERAPY FOR ADULT ACUTE MYELOGENOUS LEUKEMIA PATIENTS

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Background. CD25 represents IL-2 receptor α (IL-2Rα). CD25 antigen expression has been observed mainly in FAB-M4 and -M5 subtypes of acute myelogenous leukemia (AML). However, questions about the dependence or independence of both CD25+ leukemic cell functions in AML and the varying expressions of IL-2R for malignant myeloid cells remain unanswered. Unlike solid tumors, hematological diseases such as AML have demonstrated a wide range of soluble IL-2 receptor levels, which suggest the possibility of different patterns of proliferation of leukemic cells. In addition, AML cells possess the characteristics of cell surface CD25 inducibility. *Aims.* An attempt was made to correlate clinical outcomes with specific patterns of the expression of immune cells after induction chemotherapy (IC) in adult patients AML. *Methods.* Seventy-five newly diagnosed AML patients received the same initial IC and serial bone marrow (BM)- or peripheral blood (PB)-samples were taken. The gated CD45/CD25/CD4 cell populations were used to compare for the intensity of immunophenotypic signals and the different cell subsets, according to the treatment timeline. *Results.* As one of the best predictive prognostic parameters, patients who responded poorly to IC showed exceptionally higher levels of PB CD45+CD25+ cells on days 7 (p=0.002) and 21 (p=0.05) post-IC. The results of patients in complete remission (CR)(n=61), as well as those of the patients who showed continuous CR, showed relatively lower levels of PB CD45+CD25+ and higher CD4+CD25+ regulatory T cells in the steady PB after the standard IC, which was accurately discernible in every patient and in normal healthy individuals (n=21). We found considerably lower expression levels of BM/PB CD4+CD25+ regulatory T cells in the patients. *Conclusions.* These findings suggest that we can use the CD25+ cells during induction chemotherapy as to predict the outcome of adult AML patients. Further, it is necessary to reveal the exact function of those emerging cells during chemotherapy in the future.

0131

INHIBITION OF FLT3-ACTIVATING MUTATIONS MAY NOT PREVENT CONSTITUTIVE ACTIVATION OF ERK/AKT/STAT PATHWAYS IN SOME AML SAMPLES: A POSSIBLE CAUSE FOR THE LIMITED EFFECTIVENESS OF THERAPY WITH SMALL-MOLECULE INHIBITORS

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AML cells are characterized by genetic alterations involved in the expression of transcriptional regulators that are critical for normal hematopoietic development and differentiation. Activating mutations on Flt3 receptors are the most common genetic alterations in AML, conferring a poor prognosis and decreased overall survival. Thus, Flt3 is nowadays a promising target for therapeutic intervention, and a group of new small molecule inhibitors targeting the Flt3 RTK are currently being evaluated in clinical trials. However, clinical responses in relapsed or refractory AML are limited and transient. This study investigates the rele-

vance of activating mutations of Flt3 on the constitutive activation of intracellular pathways aberrantly active in AML, and their effect on blast cell survival. A total of 28 patients with acute myeloid leukaemia (AML) diagnosed according to the classification of the French-American-British (FAB) committee, as well as ten healthy controls, were entered into this study after informed consent. Blast cells were obtained either from peripheral blood or bone marrow aspirates at the time of diagnosis, and isolated by gradient centrifugation using Ficoll-Hypaque. Flt3 gene mutations were identified by allele-specific polymerase chain reaction (PCR) amplification from Flt3 cDNA, followed by agarose gel electrophoresis analysis. The status of activation of the MAPK/AKT/STAT pathways was analyzed by both Western blot and electrophoretic mobility shifts assays. Our results showed differential activation of multiple pathways (ERK, p38, Akt, Bcl-2, NF-κB, and STAT proteins) involved in the survival and differentiation of AML. In addition, our overall data demonstrated a differential activation of these intracellular pathways among the different AML subtypes analyzed, and even between the different AML samples belonging to the same AML subtype. Contrary to previous reports, we found that inhibition of mutant Flt3 phosphorylation did not prevent phosphorylation of ERK, STAT5 or Akt in some AML samples, suggesting the implication of Flt3-activating mutations and other cytogenetic or molecular unknown events. On the other hand, resistance to spontaneous apoptosis was found to be related to the simultaneous activation of several intracellular signals. Moreover, antiapoptotic routes regulated by the mutant and the wild-type Flt3 diverged in some subsets of primary AML blasts. Taken together our results showed differential activation of multiple pathways even in the same AML subtype, and not related to activating mutations of the Flt3 receptor. This suggests the existence of distinct mechanisms of activation or even other mutated components with constitutive activation that might silence the effect of specific kinase-inhibitors. Thus, a simultaneous intervention, adequately targeting the signaling pathways altered in each AML patient, may provide a more effective approach to reverse leukemogenesis. Supported by FIS 050910, FIS 041291 and JA 0024/2005.

0132

THE PRESENCE OF FLT3 MUTATIONS DOES NOT IMPAIR STEM CELL MOBILIZATION AND FEASIBILITY OF AUTOLOGOUS PERIPHERAL BLOOD STEM CELL TRANSPLANTATION IN ACUTE MYELOID LEUKEMIA

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Background. The presence of mutations in the juxtamembrane domain of the tyrosine kinase receptor gene fetal liver tyrosine kinase 3 (FLT3) has emerged as a powerful prognostic indicator in acute myeloid leukaemia (AML), mainly in patients with normal karyotype. Furthermore, FLT3 is preferentially expressed on the cell surface of hematopoietic progenitors, and its ligand (FL) is expressed as a membrane-bound or soluble form by bone marrow stroma cells. FL-FLT3 interaction plays an important role in the maintenance, proliferation and differentiation of hematopoiesis. Accordingly, mutations of FLT3 could play a role in mobilization of peripheral blood stem cells (PBSC) in patients with AML and, as a consequence, affect feasibility of autologous stem cell transplantation (PBASCT). *Aims.* In this study, we analyzed the relevance of FLT3 mutations on mobilization and collection of CD34 positive (CD34+) cells as well as on feasibility of ASCT from a series of 111 patients with acute myeloid leukaemia and normal karyotype. Material and *Methods.* There were 68 male patients and 43 female patients with a median age of 58 years (range 17-79). Overall, 23 patients out of 111 (21%) had FLT3 mutations. More in detail, FLT3/ITDs were detected in 14 out of 111 patients (13%), while FLT3 D835 mutations were detected in 8 of 34 patients (7%). One patient (1%) was found as having both abnormalities. *Results.* The overall CR rate of 74% and was not influenced by the presence of FLT3 mutations (64 out of 88 or 73% for FLT3- as opposed to 18 out of 23 or 78% for FLT3+ patients, *p*:0.78). The overall successful mobilization rate (> 2×10^6 CD34+ cells/kg) was of 89% (65 out of 73) and was identical for FLT3- and FLT3+ patients, i.e. 42 out of 47 for FLT3- (89%) and 16 out of 18 for FLT3+ (89%), *p*:0.69). The median number of CD34+ cells collected was 7.6×10^6/kg (range 2.1-50.8) and 7.1×10^6/kg (range 4.4-60.3) for FLT3- and FLT3+ patients, respectively (*p*:0.64). Feasibility of PBASCT was 47% for FLT3- patients (41 out of 88) as opposed to 48% for FLT3+ ones (11 out of 23), p 0.89. Among 73 patients evaluated for mobilization, feasibility of ASCT rose to 71% and, once again there was no difference between FLT3- (41 out of 55 or 74%) and FLT3+ patients (11 out 18 or 61%), *p*:0.43. Reasons for not autografting the remaining 21 patients (29%) included early relapse (n = 10), toxicity after consolidation (n = 5) and failure to mobilize PBSCT (n = 6). Of note, early relapse rate was higher for FLT3- patients (5 out of 7, or 71%) as opposed to 5 out of 14 (36%) for the group of FLT3- patients, *p*:0.27. *Conclusions.* the analysis of our data demonstrate that the presence of FLT3 mutations has no influence on mobilization and collection of CD34+ cells as well as on overall feasibility of PBASCT in AML patients with normal karyotype.

0133

MYELOABLATIVE CHEMOTHERAPY FOLLOWED BY AUTOLOGOUS STEM CELL INFUSION MAY OVERCOME THE ADVERSE PROGNOSTIC IMPACT OF FLT3 MUTATIONS IN ACUTE MYELOID LEUKAEMIA

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Background. Within the past few years the presence of an activating internal tandem duplication (ITD) in the juxtamembrane domain of the tyrosine kinase receptor gene fetal liver tyrosine kinase 3 (FLT3) has emerged as a powerful prognostic indicator, ahead of cytogenetics, predicting for relapse from complete remission (CR) in acute myeloid leukaemia (AML). In particular, patients with activating FLT3 mutations, either in the form of an internal tandem duplication (ITD) or a point mutation in the activation loop [point mutations of Asp835 (FLT3/D835 mutations)] have a significantly higher risk of relapse, namely in intermediate risk AML, in which most patients present with normal karyotype. *Aims.* To analyze the prognostic relevance of FLT3 mutations in 73 patients with acute myeloid leukaemia with normal karyotype, who survived induction and consolidation and received autologous stem cell transplantation (ASCT) after successful mobilization of peripheral blood stem cell (PBSC) in CR1. *Patients and Methods.* There were 44 males and 29 females with a median age of 54 years (range 20-77). Overall, 16 out of 73 autografted patients (22%) had FLT3 mutations. More in detail, FLT3/ITDs were detected in 10 out of 73 patients (14%), while FLT3 D835 mutations were detected in 5 cases (7%). One patient (1%) was found as having both abnormalities. Pre-transplant therapy consisted of ICE as induction (idarubicin + cytarabine + etoposide) followed by NOVIA (mitoxantrone + intermediate dose cytarabine) as consolidation and mobilizing regimen for patients aged up to 60 years, and of continuous infusion (c.i.) of fludarabine and cytarabine (ciFLA) as induction and consolidation for patients >60years. Conditioning regimen was a combination of 3 days c.i. idarubicin plus busulphan for 4 days (I-Bu) for 56 patients (reduced by one day for both drugs for patients >60 years), and classical Bu-Cy for the remaining 17 cases. *Results.* Analysis of basal characteristics of the patients showed that white blood cell count (*p:* 0.009), serum concentration of lactate dehydrogenase (*p*:0.01), and percentages of peripheral blood (*p*:0.002) and bone marrow blasts (*p*:0.03) were significantly higher in patients positive for FLT3 mutations. On the contrary, overall survival and disease free survival were similar between patients with or without FLT3 mutations (*p:* 0.73 and 0.78, respectively). *Conclusions.* Our data suggest that myeloablative chemotherapy supported by auto-PBSCT may overcome the adverse prognostic implications of FLT3 mutations in AML. However, it is to consider that autografted patients are highly selected for best response to induction, consolidation and mobilization as well as for minor non-haematologic toxicity.

0134

PEDIATRIC RELAPSED ACUTE MYELOID LEUKEMIA IN THE NETHERLANDS FROM 1980 UNTIL 2000

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The prognosis of pediatric AML has improved considerably over the past decades, with overall long-term survival rates up to 60%. However, relapse remains the major cause of treatment failure, occurring in 30-40% of patients. Patients with relapsed AML have a poor prognosis. Studies from different groups have shown survival rates of 15-30%. The outcome of Dutch pediatric relapsed AML patients is unknown. We therefore studied all pediatric *de novo* AML patients initially diagnosed

between 1980 and 1998 who suffered from a relapse. Most were initially treated on the subsequent Dutch Childhood Oncology Group (DCOG) studies ANLL 80, 83, 87 and 94. Data were collected from the central data collection center of the DCOG. From 1980-1998 354 patients were diagnosed with *de novo* AML in the Netherlands. 113 patients (32%) relapsed between 1980 and 2000. Most (63%) relapsed within a year after reaching first complete remission (CR1) (median time to relapse 9 months, range 1-56 months). 80% (N=90) of patients were treated with curative intent. Patients were treated with different treatment regimens as during most of this period no uniform treatment protocols were available. CR2 was achieved in 84% of patients after a median of 2 courses of chemotherapy. Stem cell transplantation (SCT) was performed in 22 patients after achieving CR2. Overall, probability of 10 year overall survival (10-year pOS) of pediatric relapsed AML patients was 0.16 (SE=0.04). Patients that relapsed early (CR1≤1 year) were significantly less likely to survive than patients that suffered from a late relapse (5-year pOS 0.12 vs. 0.29, $p<0.0001$). One third (8/22) underwent an autologous and two-thirds (14/22) an allogeneic SCT. We performed multivariate analysis, including SCT (as a time-dependent variable) and CR1 duration (CR1≤ 1 year). Both SCT and CR1 duration were significantly correlated to survival. Patients that received a SCT in CR2 had a significantly improved survival (RR=0.43, $p=0.008$), while a CR1 duration ≤1 year resulted in a significantly poorer survival (RR=2.7, $p<0.0001$). In conclusion, 16% of pediatric relapsed AML patients are long-term survivors. Patients with an early relapse do worse compared to patients with a late relapse, confirming results from other study groups. There is a survival benefit for SCT after relapse. We recently opened an international randomized phase III trial for children with relapsed/refractory AML (I-BFM/DCOG Relapsed AML 2001/01), randomizing FLAG (fludarabine, cytarabine, G-CSF) with or without liposomal daunorubicin. This ongoing trial enables internationally uniform treatment of the rare cases of relapsed AML in children and will show us if the addition of liposomal daunorubicin is of benefit for these children.

0135

PROTEIN KINASE CK2A AS AN INDEPENDENT PROGNOSTIC MARKER AND A NOVEL THERAPEUTIC TARGET IN ACUTE MYELOID LEUKEMIA

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Background. Protein kinase CK2 (formerly casein kinase II) is a highly conserved, and ubiquitously expressed protein serine/threonine kinase implicated in various cellular processes including proliferation, differentiation, and transformation. However, the clinical and biological significances of CK2 have not been elucidated in acute myeloid leukemia (AML). *Aims.* We tried to evaluate the clinical and biological significances of CK2 in AML, *Methods.* We first analyzed the expression and activity of catalytic subunit of CK2 (CK2α) by Western blot and its association with clinical outcomes in consecutive 59 AML patients with normal karyotype. *Results.* Western blot analyses demonstrated that CK2α expression was observed in 30 (50.8%) cases. Constitutive expression of CK2α was not demonstrated in bone marrow samples obtained from healthy volunteers. Levels of CK2α expression were highly correlated with CK2α catalytic activity ($p<0.0001$) in primary AML cells. Kaplan-Meier analysis showed that the disease-free survival (DFS) and overall survival (OS) rate were significantly lower in the CK2α-positive cases compared to the CK2α-negative cases ($p<0.05$ and $p<0.001$, respectively). Multivariate analysis revealed that CK2α expression was an independent prognostic factor in the DFS ($p=0.002$) and OS ($p=0.003$). Treatment of U937 leukemia cell line with a CK2-selective inhibitor, apigenin, for 24 h potentially reduced the expression levels of phosphorylated PTEN, phosphorylated Akt/PKB and Akt/PKB downstream molecules in a dose-dependent manner. In contrast, an induced overexpression of CK2α increased the levels of anti-apoptotic proteins including Bcl-2, Bcl-xL, Mcl-1, survivin and XIAP in U937 cells. Although apigenin did not potentially induce cell death in U937 cells, an induced over-expression of CK2α remarkably enhanced the sensitivity of the cells to the apigenin-induced cell death. Interestingly, apigenin-induced cell death was remarkably higher in the CK2α-positive primary AML samples (82±4%) compared to the CK2α-negative AML cells (4±1%, $p<0.001$), or normal BM samples (5±2%, $p<0.001$). *Conclusions.* These results strongly suggest that protein kinase CK2 is an independent prognostic marker in AML with normal karyotype. In addition, our finding that CK2 inhibitor effectively induces cell death preferentially in the CK2α-positive AML provides a novel approach to the targeted therapy for AML.

0136

INTERLEUKIN-2 CAN BE SAFELY ADMINISTERED TO AML PATIENTS IN 1 ST CR AFTER AN AUTOLOGOUS PERIPHERAL BLOOD STEM CELL TRANSPLANTATION

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Interleukin-2 (IL2) is a cytokine with anti-tumor activity. When administered after autologous stem cell transplantation, it appears to reproduce the graft versus leukemia effect of allogeneic transplant and possibly prolong disease-free survival (DFS). Since 1999, at the Hematology Department of *La Sapienza* University in Rome, 24 AML patients in Ist CR underwent immunotherapy with IL2. All patients received hydroxyurea followed by HD or SD-AraC plus Daunorubicin and Etoposide as induction treatment, and Daunorubicin plus ID-AraC as consolidation. Subsequently, an allogeneic or autologous peripheral blood stem cell transplantation (PBSCT) was planned according to donor availability. IL2 was administered following PBSCT after BU-CY conditioning regimen in 18/24 patients, and after consolidation in 6 patients not eligible for an autograft because of infections (2 pts) or mobilization failure (4 pts). IL2 therapy was started after a median of 4 months from autograft (range 1-7) and a median of 7.5 months from consolidation therapy (range 4-14). An absolute neutrophil count higher than 1x109/L, a stable platelet count greater than 50×10^9/L and no evidence of active infections were required to start the treatment. IL2 was administered subcutaneously on 5 consecutive days, on a monthly basis, for 1 year or until relapse. The dosage of IL2 was 4×10^6 IU on day 1, followed by 8x106 IU on days 2 through 5. All patients received paracetamol and prophylactic trimethoprim/sulfamethoxazole to prevent bacterial infections during IL2 therapy. No patient required treatment discontinuation because of a grade 4 toxicity according to NCI-CTC criteria. Fever (grade 1-2) was observed in all patients 4-6 hours after IL2 administration, with grade 1 arthralgia in 15 of them. The majority of patients showed gastrointestinal toxicity (grade 1-2) in the form of nausea and vomiting (21/24), diarrhoea (4/24) and transient transaminase increase (7/24). Skin toxicity (grade 1-2) was observed as desquamation (7/24), rash and pruritus which required systemic measures (4/24) and injection site reactions (16/24). With regard to hematological toxicity, grade 3 thrombocytopenia requiring a 50% dose reduction was observed in 2 patients. Concerning neurological toxicity, only 2 patients showed irritability and insomnia during IL2 administration, not requiring dose modification. No patient showed infective complications. In all cases, toxicity completely recovered within 48 hours from IL2 discontinuation. Five patients relapsed on therapy, after 2 (CNS relapse), 3, 4, 6 and 11 months from the start of IL2, while 2 patients relapsed after 5 and 13 months from treatment discontinuation. One patient is still on therapy and 16 are in CCR after a median of 18 months (range 1-63) from IL2 discontinuation. The eighty-months projected probability of DFS for the 24 patients is 55%, the median has not been reached. Based on our experience, it appears that IL2 therapy is a feasible approach devoid of serious toxicity also after an autograft procedure. A randomized trial is currently ongoing in the context of the EORTC/GIMEMA AML12 protocol to document whether or not IL2 is capable to enhancing the likelihood of disease-free survival.

0137

STEM CELL DETECTION IN AML PATIENTS UNDER REMISSION CONDITIONS: A NEW ERA IN MINIMAL RESIDUAL DISEASE DETECTION?

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Background. In CD34-positive acute myeloid leukemia (AML), the leukemia-initiating event likely occurs in the CD34+CD38- stem cell-compartment. Survival of these cells after chemotherapy hypothetically leads to minimal residual disease (MRD) and relapse. *Aims.* In the present study we investigated, using 4-colour flowcytometry, whether aberrant antigen expression at diagnosis can be used to identify AML CD34+CD38- cells in remission bone marrow. Such would offer opportunities for stem cell MRD detection and for future patient risk stratification and guidance of therapeutic intervention. *Methods.* FACS analysis was performed on fresh bone marrow CD34+CD38- cells of AML samples at diagnosis and after chemotherapy and of normal and regenerating bone marrow samples. Antibody combinations always consisted of anti-CD34FITC, anti-CD45PerCP and anti-CD38APC together with different PE-labelled antibodies against CLL-1, CD2, CD5, CD7,

CD11b, CD19 and CD56, with the exception of CLL-1 all derived from leukemia-associated phenotypes, LAP's, used in immunophenotypic MRD detection (Feller *et al.*, Leukemia 8:1380, 2004). Expression on CD34+CD38- cells was scored as <50% or >50%. At least 50% expression at diagnosis is needed for accurate measurements of residual malignant cells after chemotherapy. *Results.* We found that at diagnosis (60/77) AML samples of a consecutive cohort showed >1% CD34+ cells and were considered as CD34-positive. We were able to measure a reliable number of CD34+CD38- events (>20) in 56/60 cases. CLL-1 expression was >50% in 15/60 cases, LAP expression in 9/60 cases and both CLL-1 and LAP in 8/60 cases. Altogether in 32/60 CD34-positive cases, AML stem cell MRD was possible. In normal bone marrow (n=4) as well as in regenerating bone marrow, the CD34+CD38- cells did show low (<3%) CLL-1 expression (n=6) and low LAP expression (n=2, for all antigens). Therefore, under MRD-conditions CLL-1 and/or LAP staining might enable to accurately discriminate between normal and malignant CD34+CD38- cells in part of AML patients. In agreement with this, the different ratios of malignant and normal CD34+CD38- cells, that were found in a number of patients in follow-up material paralleled clinical outcome. For comparison, *whole blast* MRD measurements using LAP was possible in 51/60 CD34-positive patients. In 3 patients without *whole blast* MRD possible, stem cell MRD could be used. The higher success rate for *whole blast MRD* is partly due to the absence of a detectable CD34+CD38- population in some patients, and to the possibility to detect LAPs on the CD34 negative compartment in the CD34 positive AML. However, the success of stem cell MRD will increase using antibody combinations known as LAP, but which need at least 5 fluorescence channels. The main improvement in stem cell MRD will likely come from the introduction of the alternative stem cell population, ie the side population (SP). In 7/7 AML samples studied thus far and including both CD34-positive samples and CD34-negative samples, SP cells were present and shown to have LAP/CLL-1 expression in all cases (see abstract Moshaver *et al.*). Lastly, since the population of interest is so well defined [CD34+/CD38-/CD45dim/SSClow/aberrant marker(s)], stem cell MRD may require much less extensive experience than *whole blast MRD*.

0138

ANALYSIS OF X-CHROMOSOME INACTIVATION PATTERNS IN IRANIAN PATIENTS WITH AML DURING REMISSION

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Background. Analysis of x-chromosome inactivation patterns in female patients have been used to assess clonality of various tumours and X-linked disorders. Conflicting results have been published on the frequency of clonal patterns in female patients with Acute Myeloid Leukemia (AML). Previous studies have used DNA methylation to measure X inactivation, but aberrant methylation is known to occur in some situations. *Aim.* The aim of this study was to evaluate the patterns of X-chromosome inactivation during the remission in AML patients at the RNA level. *Materials and Methods.* Two hundred normal females and 45 patients with AML at remission were selected. A non radioactive reverse transcription polymerase chain reaction (RT PCR) method was used to study the expression of the polymorphisms of G6PD, iduronate 2 sulfatase (IDS) and palmitoylated membrane protein(P55) genes at the RNA level. *Results.* The frequency of heterozygosity was found to be 48.5% (119/245) for P55 gene. Forty percent (93/245) were heterozygous for IDS and only 28.9% (71/245) of individuals showed polymorphism at nt.1311 C/T for G6PD gene. Some individuals were heterozygous for more than one gene polymorphism. 92/100 (92%) normal female individuals showed a polyclonal X-chromosome inactivation pattern in lymphocytes (L) and granulocytes (G). Clonal patterns were observed in lymphocytes and granulocytes of 44/45 (98%) *de novo* AML patients at presentation, a significantly higher proportion than in controls (8%) (p<0.01). 23/27 (85.2%) of patients at remission had a clonal X-chromosome inactivation pattern in both G and L cells. 4/27 (15%) patients showed polyclonal patterns. Ten patients were available for a longer follow up. A clonal pattern was observed in G, L and T cells of seven patients. Three patients converted from clonal to polyclonal and showed a polyclonal X-chromosome inactivation pattern in G, B and T lymphocytes. *Conclusion.* From this study, it can be concluded that clonality at remission is a frequent event in AML and does not necessarily mean relapse of the disease. There is also a possibility of the conversion of clonality to polyclonality by time.

0139

PROGNOSTIC ROLE OF EOSINOPHILIA AND CYTOGENETICS ON TREATMENT RESPONSE AND SURVIVAL IN AML-M4. THE GIMEMA EXPERIENCE

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Background. The acute myeloid leukemia (AML)-M4 subtype is frequently associated to eosinophilia and/or to the cytogenetic alteration inv(16)/t(16;16). The presence of these features is generally associated with good prognosis, but the studies concerning their exact role are hampered by the low number of cases. *Aims.* To assess the influence of eosinophilia and of the inv(16) on the prognosis of acute myelomonocytic leukemia (M4) and acute myelomonocytic leukemia with abnormal eosinophils (M4Eo). *Methods.* In a non concurrent-prospective setting, we analyzed patients with AML-M4 consecutively enrolled in two GIMEMA clinical trials, in 35 Italian hematological divisions. *Results.* Between December 1993 and December 2002, 1686 valuable adult patients over 1702 with a diagnosis of AML, were consecutively admitted to the EORTC-GIMEMA AML10 and AML 99p trials; among these, 400 patients (355 M4 and 45 M4Eo) were studied. The diagnosis of M4 and M4Eos was first established at each institution and subsequently centrally reviewed at the time of study entry. The following parameters were evaluated: morphology, immunophenotype, cytogenetics performed at the onset of the disease, complete remission achievement and duration, overall survival (OS) and disease-free survival (DFS) from AML diagnosis. Cytogenetic analysis failed or was not carried out in 40% of cases, while it was successfully analyzed in 240 cases; inv(16) was found in 17% of them. Patients with M4Eo were younger and more frequently associated with inv(16) compared to M4. Concerning the probability of obtaining a CR after standard treatment, at univariate analysis M4Eo had a trend significant advantage compared to M4, while presence of inv(16) was significantly correlated to a higher CR probability; the proportion of patients with resistant disease was higher in patients with M4 morphology compared to M4Eo. Fitting a statistical model for the analysis of factors including interactions, the multivariate analysis showed a significant advantage only of M4Eo + inv(16) compared to M4-without eosinophilia and without inv(16). DFS was not different in univariate analysis between patients carrying or not inv(16), while a borderline advantage of M4Eo was observed with respect of M4, not confirmed at multivariate analysis. OS curves showed at univariate analysis a significant advantage both of the presence of eosinophilia (p=0.004) and of inv(16) (p=0.01); at multivariate analysis, patients with M4Eo+ inv(16) had a highly significant advantage compared to M4 without eosinophilia and without inv(16) (p=0.004), but also compared to M4 + inv(16) (p=0.043), and M4Eo-without inv(16) (p=0.076). Finally OS and DFS of the 400 patients with M4and M4Eo was compared to the general AML population with different FAB: the median duration was of 19.0 and 16.5 months respectively for OS (p=0.17) and 19.7 and 16.4 months for DFS (p=0.51) in M4 versus other AML. *Conclusions.* AML-M4 with or without eosinophilia represent 23.7% of AML. The presence of eosinophilia and of inv(16)/t(16;16) can be both considered favourable prognostic factors; however, only the association of both features allows a highly significant advantage in terms of CR and OS. Concerning OS the combination of both is also significant vs. the presence of one of them.

0140

POLYMORPHISMS IN THE RAD51 AND XRCC-3 GENES INCREASE THE RISK OF DEVELOPING ACUTE MYELOID LEUKAEMIA

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Background. DNA is at constant risk from damage from both endogenous and exogenous sources and this damage causes chromosomal instability leading to oncogenesis, apoptosis and severe failure of cell functions. DNA is protected from damage by detoxification enzymes belonging to two different classes or damage triggering phases: phase I enzymes metabolise the exogenous agent to a reactive state, whereas phase II

enzymes detoxify the reactive intermediate by catalysing conjugation. Even if greater activation and lesser detoxication of carcinogens result in DNA adducts, it is still possible for genomic integrity to be restored through DNA repair. Several polymorphisms in genes involving in both detoxification and repair pathways have been identified and many of them have been shown to influence the risk of developing solid tumours and haematological malignancies. *Aims.* the aim of our study is to investigate the frequency of polymorphisms involved in detoxification and double strand break (DSB) repair via homologous recombination (HR) pathways and to correlate them with AML or therapy-related AML (t-AML) risk. *Methods.* we studied 160 patients with AML (132 *de novo* and 28 therapy-related) and 134 control subjects, matched for age and sex. RFLP PCR were used to analyze genotypes of DNA repair genes for RAD51 (RAD51-G135C) and XRCC3 (XRCC3-Thr241Met) and detoxification genes for NQO1 (NQO1-Pro187Ser) and GSTA1 (GSTA1 promoter region, *A/*B). The polymorphism in the promoter region of detoxification gene CYP3A4 (CYP3A4-A290G) was examined by mismatch PCR. *Results.* When comparing AML patients to controls, a statistically higher prevalence of the g/c + c/c genotype of the DNA repair enzyme RAD-51 was found in AML patients (22% versus 12.7%,O.R. 1.9, 95% C.I. 1-3.6, p=0.04), in particular when associated to the CYP3A4-A290G detoxification enzyme polymorphism. This was confirmed by the multivariate analysis (p=0.047 for the association). Similarly the homozygous met/met mutant of XRCC3 was more frequent in AML patients (24% vs 12%, OR 2.3, 95% C.I. 1.2-4.3). No differences were found when looking at NQO1, GSTA1 and CYP3A4 polymorphisms, alone or in association to XRCC3. In the AML patient group, we found no associations between enzymatic polymorphisms and type of AML (*de novo* versus therapy-related). *Summary/Conclusions.* DNA repair enzymatic polymorphism in the RAD51 and XRCC3 genes may increase the risk of developing acute myeloid leukaemia. This risk is particularly high when the RAD51-G135C DNA repair polimorphism is associated to the CYP3A4-A290G detoxification enzyme polymorphism.

Genotype	Controls n (%)	AML n (%)	O.R.	95% C.I.	p
XRCC3-241 *Thr/Thr + Thr/Met Met/met*	118 (88.06) 16 (11.94)	121 (76.10) 38 (23.90)	2.3	1.2-4.3	**0.01**
RAD51 *g/g g/c + c/c*	116 (87,22) 17 (12.78)	124 (77.99) 35 (22.01)	1.9	1-3.6	**0.04**
CYP3A4-A290G *a/a a/g + g/g*	121 (96.80) 4 (3.20)	135 (91.22) 13 (8.78)			0.07
NQO1 *Ser/Ser Ser/Pro + Ser/Pro*	84 (70.59) 35 (29.41)	99 (64.29) 55 (35.71)			0.3
GSTA1 **A/*A *A/*B + *B/*B*	49 (41.52) 69 (58.48)	64 (43.54) 83 (56.46)			0.7

0141

THE ANTI-PROLIFERATIVE AND APOPTOSIS-INDUCING EFFECTS OF THE PROTEASOME INHIBITORS BORTEZOMIB AND PR-171 IN ACUTE MYELOGENOUS LEUKAEMIA

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Background. Proteasome inhibitors represent a new class of anti-neoplastic drugs with documented effects in multiple myeloma and mantle cell lymphoma. *in vitro* studies suggest that these drugs also have effects on other haematological malignancies. *Aims.* The aim of this study was to investigate the *in vitro* effects of proteasome inhibitors on human primary acute myelogenous leukaemia (AML) blast proliferation, viability/apoptosis induction and clonogenic potential. Furthermore, possible correlations with genetic features of AML cells, such as Flt3 mutations and cytogenetic abnormalities were assessed. *Methods.* Native human AML blasts from peripheral blood of more than 50 consecutive patients were analysed. The impact of proteasome inhibition was examined in the following experimental models: - Cell proliferation: Leukaemia cells were cultured *in vitro* in the presence of exogenous growth factors (IL-3, SCF, GM-CSF) for 7 days and cell proliferation was measured by either [3H]-thymidine incorporation or a colony formation assay. - Cell viability: Leukaemia cells were cultured in the presence (7 days) or absence (2 days) of exogenous cytokines and apoptosis/viability was measured by flow cytometry analysis of Annexin-V expression/Propidium Iodide exclusion. - Cytokine secretion: ELISA and Multiplex assays for CXCL10, CCL3, CXCL8, GM-CSF, IL-1β, IL-6 and TNFα. - Proteasome activity; release of the fluorophore 7-amino-4-methylcoumarin (AMC) from N-Suc-Leu-Leu-Val-Tyr-AMC. We investigated the reversible proteasome inhibitor bortezomib (Velcade®) and a novel epoxomicin derivative, PR-171, which is an irreversible inhibitor (Demo SD, *et al.* Biochemical and cellular characterization of the novel proteasome inhibitor PR-171 [abstract]. Blood 2005; 106:455a). *Results.* Basal proteasome specific activity in AML blasts varied 1-10 fold. Both drugs inhibited the proteasome complex with equivalent potency and suppressed cell viability as determined by annexin/PI staining after 48 hr compound treatment (% Annexin/PI negative cells; mean with standard deviation: control 33±15; bortezomib 25 nM 9±5 and 50 nM 12±11; PR-171 25 nM 9±5 and 50 nM 8±9). The drugs also inhibited AML cell proliferation as measured by incorporation of [3H]-thymidine after 7 days of compound treatment (IC50 95% confidence intervals: bortezomib 20.99-24.78 nM; PR-171 9.76-10.43 nM). Cytotoxic and antiproliferative responses of blasts to proteasome inhibition were heterogeneous, but independent of cytogenetic abnormalities, Flt3 mutations, FAB classification or CD34 expression. A subset of AML patient samples exhibited greater sensitivity to PR-171 relative to bortezomib in both [3H]-thymidine incorporation and colony formation assays. Both drugs modulated the constitutive cytokine release by human AML cells. *Conclusion.* Our studies show that proteasome inhibitors have dose-dependent and marked effects on proliferation, viability and colony forming properties of human native AML blasts at nanomolar levels *in vitro*.

0142

SINGLE CELL ANALYSIS OF PHOSPHOINOSITIDE 3-KINASE/AKT AND ERK ACTIVATION IN ACUTE MYELOID LEUKEMIA BY FLOW CYTOMETRY

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Background. Acute myeloid leukemia (AML) is an aggressive malignancy and new therapeutic agents are needed. Abnormal activation of several signal transduction pathways such as phosphoinositide 3-kinase (PI3K) and MAP kinase has been reported in AML. To test new targeted therapeutics, it is critical to develop sensitive analytical tools to detect abnormal activation of these pathways and to monitor their inhibition in response to treatment. *Aims.* Our aim was to establish the feasibility of a flow cytometry analysis in patients samples even with a low blast infiltration and to analyze the correlation between flow cytometry and western blot analysis (WB). *Methods.* We analyzed AKT and ERK phosphorylation in blast cells of 72 patients with *de novo* AML. For 32 patients with high blast infiltration we compared WB and flow cytometry techniques. The flow cytometry protocol associated intra cellular staining for phospho proteins and membrane staining for several antigens including CD45 and CD34. Using CD45 allowed to identify the blast cell population even in CD34 negative AML (one third of the patients in our experience). For the CD34 positive AML, we set up a four color protocol with CD34, CD38 and CD123 as membrane antigens. This protocol allowed us to detect phosphorylated proteins in the most immature leukemic cells with the CD34+ CD38-/low CD123+ phenotype. *Results.* The correlation between flow cytometry and WB was excellent. In our series we detected PI3K and ERK pathway activations in 45% and 70% of the samples. Flow cytometry allowed the analysis of samples that were not suitable for WB analysis (low material amount) and of samples with low blast infiltration that were not interpretable with WB. In the positive samples, we could identify an immature blast cell population among the whole leukemic bulk that already harbored PI3K and ERK activation. *Conclusions.* Flow cytometry is a fast and reliable method for the detection of constitutive phospho AKT and phospho ERK in leukemic samples. All samples can be analyzed with a protocol using CD45. We analyzed the phosphorylation status of the PI3K and ERK pathways in the most immature blast cells with the CD34+ CD38-/low CD123+ phenotype. When we detected phosphorylated proteins in the whole blast cell population, this activation was already present in the most immature cells, that represent exquisite target cells for new therapeutics.

Chronic myeloid leukemia I

0143

ABERRANT EXPRESSION OF CELLULAR RETINOL-BINDING PROTEIN-1 (CRBP-1) IN MEGAKARYOCYTES AND MARROW STROMA CELLS OF CHRONIC MYELOPROLIFERATIVE AND MYELODYSPLASTIC/MYELOPROLIFERATIVE DISORDERS

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Background. The effects of retinol (ROL) are mediated by cytoplasmic binding proteins involved in retinoid transport and/or metabolism, as well as nuclear receptors which act as ligand-dependent transcriptional regulators. Cellular retinol-binding protein (CRBP) -1 contributes to the esterification of ROL to retinyl esters, the oxidation of ROL to retinal, the hydrolysis of retinyl esters into ROL. It also has been implicated in cellular growth and differentiation. Lipid droplets of hepatic stellate cells of normal and fibrotic liver which are the main storage site for retinoids contain high amounts of CRBP-1. Moreover, CRBP-1 is widely expressed in many extrahepatic vitamin-A target tissues including normal prostate, breast, endometrial glands and stroma, and cervical epithelium. *Aims.* Heterogeneous patterns ranging from over-expression of CRBP-1 to down-regulation via epigenetic silencing through DNA hypermethylation has been reported in several malignancies. To investigate the involvement of this key protein of retinoid homeostasis and metabolism in myeloproliferative diseases (MPD) and in myelodysplastic/myeloproliferative overlap syndromes (MDS/MPD), we analysed the *in situ* expression patterns of CRBP-1. *Methods.* This study was performed on a cohort of healthy bone marrow donors (n=15), patients with essential thrombocythemia (ET; n=25), chronic idiopathic myelofibrosis (CIMF; n=25), polycythemia vera (PV; n=25) and in MDS/MPD with features of so-called essential thrombocythemia with ringed leukemia (ET/RS; n=70). The tissue localization of CRBP-1 in marrow trephines was visualized using a well characterized antibody. Imaging was performed by bright-field and confocal laser scanning microscopy (CLSM). Double-labeling experiments included a panel of antibodies such as CD6,1 CD34 or a-smooth muscle actin (SMA). Evaluation focused on CRBP-1 expression in megakaryocytes and bone marrow stromal cells/myofibroblasts (MSCs/MFs). CRBP-1+ MSCs/MFs were present in subsets of MPD patients, but not in normal controls and increased from praefibrotic CIMF to CIMF III. Colocalization of CRBP-1 and SMA was documented by CLSM. *Results.* The up-regulation of CRBP-1 in MSCs/MFs was associated with an increased fibre density in the various MPD entities including CML, CIMF and PV, but not in ET. Bone marrow stromal cells from a subset of patients presenting with high platelet counts and ringed sideroblasts exhibited traits of MSCs/MFs which were similar to classical CIMF. Megakaryocytes from healthy control persons showed a moderate to high cytoplasmic CRBP-1 immunoreactivity. In contrast to the stroma, heterogeneous levels were demonstrated in megakaryocytes of PV and subsets of ET. CRBP-1 loss or abnormal spotty localization was most prominent in the bizarre giant megakaryozytes of CIMF while smaller megakaryocytes of CIMF showed a stronger cytoplasmic immunolabeling. Similar patterns of CRBP-1 expression were observed in a subset of patients with ringed sideroblasts with bizarre megakaryocytes. *Conclusions.* The modulation of CRBP-1 in MSCs/MFCs of the marrow microenvironment may affect proliferation, migration, differentiation, matrix synthesis and turnover in MPD. Moreover, the retinoid-signaling cascade may be impaired in megakaryocytes. Aberrant regulation may occur as a consequence of point mutations or a disruption of the ordered pattern of DNA methylation. Until now, the molecular mechanisms affecting CRBP-1 in MPD and MDS/MPD overlap syndromes remain to be identified.

0144

CHRONIC MYELOID LEUKEMIA CELLS EXPRESS TUMOR ASSOCIATED ANTIGENS ELICITING SPECIFIC CD8+ T CELL RESPONSES AND ARE LACKING COSTIMULATORY MOLECULES

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Background/Aims. Specific immunotherapies for patients with chronic myeloid leukemia (CML) might eliminate residual CML cells after therapy with imatinib or chemotherapy, and might enhance a specific graft versus leukemia effect after allogeneic stem cell transplantation. *Methods.* Here, we investigated the mRNA expression and T cell recognition of tumor/leukemia-associated antigens (TAAs/LAAs) in 34 patients with CML. *Results.* Several LAAs are expressed in CML and therefore candidate structures for specific immunotherapies: bcr-abl (100%), G250 (24%), hTERT (53%), MPP11 (91%), NEWREN60 (94%), PRAME (62%), Proteinase3 (71%), RHAMM/CD168 (83%) and WT1 (53%), but not BAGE, MAGE-A1, SSX2 or NY-ESO-1. The frequency of mRNA expression of RHAMM/CD168, Proteinase3 and PRAME was higher in acceleration phase and blast crisis. In flow cytometry, CD34+ progenitor cells typed positive for HLA-molecules, but were deficient for CD40, CD80, CD83 and CD86. However, RHAMM/CD168 R3-peptide (ILSLELMKL) specific T cell responses in CML patients were demonstrated by ELISPOT analysis and specific lysis of RHAMM/CD168 R3-pulsed T2 cells in chromium-51 release assays. RHAMM-R3 specific T cells could be phenotyped as CD8+tetramer+CD45RA+CCR7-CD27- early effector T cells by tetramer staining. *Conclusion.* Therefore, vaccination strategies inducing such RHAMM-R3 directed effector T cells might be a promising approach to enhance specific immune responses against CML cells.

0145

MOLECULAR RESPONSE AT 6 MONTHS IS A GOOD EARLY PREDICTOR OF DURATION OF TREATMENT RESPONSE IN IMATINIB-TREATED CHRONIC PHASE CHRONIC MYELOID LEUKAEMIA

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Background. Imatinib mesylate induces a complete cytogenetic response (CCR) in >75% of *de novo* chronic phase CML patients. Monitoring of disease response with highly sensitive real-time quantitative reverse-transcriptase PCR (RQ-PCR) provides prognostic information in addition to that obtained by cytogenetic monitoring. Achievement of a 3-log reduction in BCR-ABL/BCR from standardised baseline (major molecular response, MMR) on imatinib confers improved progression-free survival and occurs in 40% of patients treated with 400 mg daily for one year (IRIS study). *Aims.* The TIDEL study aimed to assess the effect of imatinib dose escalation on treatment outcome. The starting dose was 600mg daily. Where possible, dose-escalation to 800 mg daily was undertaken for failure to achieve complete haematologic response (CHR) at 3 months, major cytogenetic response (MCR) at 6 months, CCR at 9 months or 4-log reduction in BCR-ABL/BCR at 12 months. The principal clinical outcomes of TIDEL will be reported elsewhere. In this sub-study we examined early molecular response in TIDEL patients as a predictor of subsequent MMR and event-free survival (EFS). *Methods.* Newly diagnosed CML patients in chronic phase were treated with imatinib in a single-arm prospective cohort study and monitored with RQ-PCR in a central laboratory. Molecular response was reported according to log-reduction in BCR-ABL/BCR ratio from a standardised baseline. MMR was confirmed with two consecutive *Results.* The Australasian Leukaemia and Lymphoma Group treated 103 patients with a median follow-up of 30 months. CCR was achieved in 94 patients (91%) after a median of 3 months with a median 2.2-log reduction at the time of CCR. MMR was achieved in 66 patients (64%) after a median of 9 months (range 3-29). Defined events for loss of response occurred in 19 patients (18%) with loss of CCR (n=11), loss of MCR (n=2), loss of CHR (n=3), accelerated phase (n=1) or blast crisis (n=2). Four patients died without loss of response (2 myocardial infarctions, 1 suicide, 1 transplant-related) and were excluded from EFS analysis. We examined molecular response at 3 and 6 months, and at the time of first documented CCR as early predictors of MMR and EFS. At all times there was an association between molecular response and subsequent achievement of MMR (see Table).

Table 1. MMR and EFS according to molecular response.

Time	BCR-ABL/BCR log-reduction	(%)	% achieving MMR	P value	EFS at 30 mo (%)	P value
3 mo	<2	63	52	<0.01	76	NS
	>=2	37	88		88	
6 mo	<2	33	36	<0.01	62	<0.01
	2-<3	36	68		96	
	>=3	31	100		100	
CCR	<2	42	54	<0.01	81	NS
	2-<3	43	76		84	
	>=3	16	93		100	

Only molecular response at 6 months was significantly associated with EFS at 30 months: <1-log reduction 40%; 1-<2-log reduction 66%; 2-<3-log reduction 96%; ≥3-log reduction 100%. The degree of log-reduction in BCR-ABL/BCR at 3 months, and at the time of CCR was not predictive of EFS. *Summary.* In this study where achievement of CCR is accelerated (in comparison with IRIS), molecular response at the time of detecting CCR lacks prognostic value with regard to EFS, highlighting the importance of molecular monitoring at regular intervals, rather than waiting until CCR is achieved. Despite excellent treatment responses overall, with early molecular monitoring it is possible to identify a group of patients whose chance of achieving MMR is low, and in whom closer monitoring or alternative treatments should be considered.

0146

BCR-ABL PEPTIDE VACCINATION INDUCES ADDITIONAL MOLECULAR IMPROVEMENT IN IMATINIB-RESPONSIVE CHRONIC MYELOID LEUKAEMIA

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CML is characterised by the fusion BCR-ABL protein. The unique amino acid sequences KQSSKALQR and GFKQSSKAL spanning the e14a2 (b3a2) fusion junction may be expressed on CML cells. In our phase I/II Evaluation of Peptide Immunisation in CML (EPIC), the vaccine consisted of 3 peptides containing 9 and 13-mer sequences spanning the e14a2 BCR-ABL junction, both unmodified and also linked to the 15-mer pan HLA-DR epitope (PADRE; to which all subjects are immunologically naive). All 18 entrants were in complete haematological response in first chronic phase with at least 8 months prior treatment with imatinib. Each patient received 6 vaccinations over 2 months, at one of 4 escalating peptide doses between 100 and 1000 ug. Here we report the molecular effects of vaccination. Responses were assessed at baseline, 3 weeks, monthly from 2 to 6 months and then 3-monthly. The BCR-ABL transcript level was expressed as a BCR-ABL/ABL ratio, measured using real-time quantitative RT-PCR on a LightCycler. The immune response to the BCR-ABL peptides and PADRE were tested using IFN-γ ELISPOT assays at the same time points. No difference in molecular responses was seen between the 4 dose cohorts. At 6 months after commencing vaccination (i.e. 4 months after completion of the final vaccination), 13 cases showed a reduction in their BCR-ABL levels (to less than 5%). In 11 of these, the molecular improvement was detected 1-2 months after an immune response to BCR-ABL was first evident. In these 11 cases, their BCR-ABL levels fell to a median of 19% (range 1%-38%) of the pre-vaccination level. In 2 of the 13 molecular improvers, no immune response was detected to BCR-ABL at any time point and their BCR-ABL/ABL levels were only reduced to 71-73% of the starting level. At 6 months after commencing vaccination, 5 cases had no reduction in their BCR-ABL levels, and these were the patients with no pre-vaccination molecular response to imatinib. It is possible that the fall in BCR-ABL seen in the molecular improvers was due to an ongoing response to imatinib. In order to investigate this, the mean 3-monthly log reduction in BCR-ABL level over the 6 months prior to commencing vaccination was calculated (*pre-EPIC*). Similarly, the mean 3-monthly log reduction over the 15 months after vaccination was calculated (*post-EPIC*). Of 12 assessable cases, 7 had received imatinib for more than 12 months pre-vaccination. In 6 of these 7, the post-EPIC BCR-ABL fall is greater than the pre-EPIC fall. Conversely, among the 5 cases who had received imatinib for less than 12 months pre-vaccination, the pre-EPIC BCR-ABL fall was greater than that post-EPIC in 3 cases. In summary, the data suggest that the BCR-ABL peptide vaccination can generate an immune response which leads to the reduction of BCR-ABL in some CML patients. The vaccination strategy appears most effective in patients who have already responded well to at least 12 months of imatinib, where it may provide additional molecular control over that achievable by imatinib alone. However, vaccination may be ineffective in patients who have failed imatinib.

0147

P210 BCR-ABL TYROSINE KINASE INTERACTS WITH HISTONE DEACETYLASE 1 IN CHRONIC MYELOID LEUKAEMIA HAEMATOPOIETIC PROGENITORS: CONSEQUENCES ON HISTONE H4 ACETYLATION AND CHROMATIN STRUCTURE

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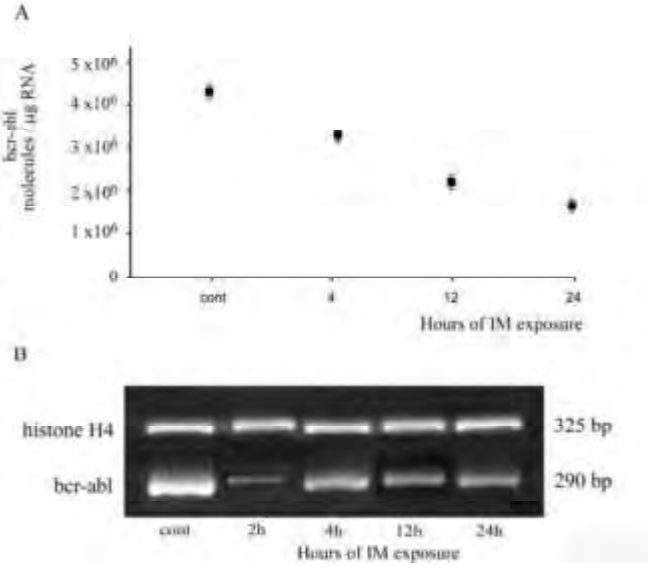
The BCR-ABL fusion gene originated from balanced (9;22) translocation is the molecular hallmark and the causative event of Chronic Myeloid Leukaemia (CML). The interactions of its p210 protein constitutively activated and improperly confined to the cytoplasm with multiple regulatory signals of cell cycle progression, apoptosis and self-renewal induce the illegitimate enlargement of clonal hematopoiesis and genetic instability that drives its progression towards the fully transformed phenotype of blast crisis. However, its effects on the basic transcription machinery and chromatin remodeling are unknown. Our study underscored histone H4 hyperacetylation associated with p210 tyrosine kinase (TK) *in vitro* and *in vivo* and its role in BCR-ABL transcription. Histone H4 acetylation status was assayed in 32D murine myeloid progenitor cell line expressing a ts BCR-ABL mutant by labelling immunoprecipitated (IP) chromatin (CHIPs) with an anti-Ac-H4 antibody. Under permissive culture condition for p210 TK (33°C), histone H4 acetylation was reduced between 4 and 24 h of imatinib mesylate (IM) exposure concomitantly with p210 dephosphorylation and enzimatic activity reduction. *in vivo* histone H4 acetylation signals on CHIPs of CD34+ progenitors from CML patients at diagnosis were more intense than those of normal controls and were significantly reduced at day 15 of IM therapy. To address the putative p210 TK role on histone H4 methylation *in vitro* and *in vivo* advanced by mass spectrometry analyses we proved that histone H4 trimethylation at Lys20 was significantly reduced in presence of p210 TK and restored after p210 TK inhibition by IM *in vitro*. Histone H4 hyperacetylation associated with p210 TK *in vitro* and *in vivo* proceed, at least in part, from Hdac1 loss of function arising from its cytoplasmatic compartmentalisation by p210 TK. Indeed p210 TK is associated with histone H4 hyperacetylation at a BCR promoter region (-40 to +285) critical for BCR-ABL transcription in LAMA cell line. BCR-ABL transcript levels were reduced by approximately 20% at 4 h of IM exposure and further declined to 40% of untreated control at 24 h. Amplification signals of DNA from anti-Ac-H4 CHIPs were significantly reduced at 2 h of IM exposure and remained lower compared with untreated control up to 24 h (Figure 1, part A and B). Complementary activities are probably implicated in the control of histone H4 acetylation status relative to p210 TK.



0148

BCR/ABL ONCOGENIC KINASE DISRUPTS MISMATCH RECOGNITION AND REPAIR COMPLEX TO INDUCE GENOMIC INSTABILITY

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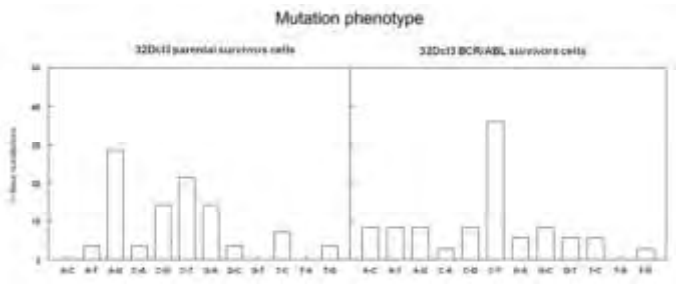
Background. BCR/ABL oncogenic tyrosine kinase is present in most chronic myeloid leukemia (CML) and in a cohort of acute lymphocytic leukemia (ALL) patients. BCR/ABL is responsible for malignant transformation of hematopoietic cells rendering them independent of their environment. The other, less understood, role of BCR/ABL in haematological malignancies is deregulation of DNA damage response, which results in drug resistance and genomic instability. Mismatch repair proteins (MMR) are responsible for detecting and removing misincorporated nucleotides, which escaped proofreading activity of DNA polymerases. MMR proteins assembled on the mismatch can signal to repair or apoptosis. Defects in expression of MMR genes leads to drug resistance and mutator phenotype, observed in different solid tumors. *Aims.* Deciphering the role of mismatch repair in drug resistance of BCR/ABL-transformed cells. *Methods.* MNNG (N-methyl-N-nitro-N-nitrosoguanidine), methylating agent was used as a genotoxic treatment. 32Dcl3 cells myeloid cell line along with p210BCR/ABL expressing counterparts and primary leukemia and normal bone marrow cells were employed. Cell viability was assessed by trypan blue exclusion and/or propidium iodide staining. Clonogenicity of parental and BCR/ABL cells after MNNG challenge was examined in semi-solid medium. Protein expression was analyzed by Western blotting. Immunofluorescence analysis of the nuclear localization of MMR proteins was performed with primary antibodies to MSH2, MSH6, MLH1 and PMS2. Mutation rate and phenotype was analyzed using TA cloning kit and sequencing. *Results.* Among different genotoxic agents, BCR/ABL cells were more resistant to MNNG than parental cells (as shown in viability and clonogenic tests). Parental cells and BCR/ABL expressing clones were incubated with MNNG for 4 weeks resulting in their MNNG-resistant derivatives, which may accumulate mutations in their genomic DNA resulting from methylating activity of the drug. To investigate the mutation rate and phenotype, ouabain-resistance test was employed. The clonogenic assay revealed over 5 times more ouabain resistant colonies in MNNG-resistant BCR/ABL-positive cells than in parental counterparts. The dominating mutation in BCR/ABL MNNG-resistant cells was C to T, while A to G mutations was prevalent in parental cells (Figure 1). In order to check the status of MMR proteins, Western blotting and immunofluorescence studies were performed. Expression of MMR proteins in BCR/ABL transformed cells was similar to parental, however immunofluorescence visualized dramatic changes after DNA damage in the nuclear co-localization of MMR proteins in BCR/ABL-transformed in comparison to normal cells. Co-localization of MSH2 and MSH6 proteins, forming a heterodimer homologous to bacterial MutS, remained similar in parental and leukemia cells upon MNNG treatment. However, co-localization of MLH1 (which form a heterodimer with PMS2 homologous to bacterial MutL) and MSH2 was detected in non-transformed cells, but not in BCR/ABL leukemia cells. Interaction of MMR proteins in leukemia cells was restored after inhibition of BCR/ABL kinase by imatinib. *Summary/Conclusions.* BCR/ABL impairs assembly of MMR proteins on mismatched nucleotides and subsequent signaling to repair and/or apoptosis. These results suggest a novel mechanism how oncogenic tyrosine kinase can modulate mismatch recognition and repair leading to genomic instability and drug resistance of leukemic cells.



0149

COMPARATIVE PROTEOMIC ANALYSIS OF CHRONIC MYELOGENOUS LEUKEMIA CELLS : INSIDE THE MECHANISM OF IMATINIB RESISTANCE

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Background. Development of imatinib resistance represents a critical factor for the therapy of chronic myelogenous leukaemia (CML). Resistance is mainly due to mutations in the abl kinase domain, and to overexpression of Bcr/abl protein, provoked by amplification of the genomic locus. *Aims.* We undertook a comparative proteomic approach of human chronic myeloid leukemia cells Imatinib sensitive and resistant, to dissect the molecular mechanism of resistance. In fact, the characterisation of biochemical pathways involved with and connected to Bcr/abl could be extremely useful in identifying new therapeutic targets to bypass resistance to the kinase inhibitor. *Methods.* Total cell protein LAMA 84-S and LAMA 84-R extracts were separated by two dimensional electrophoresis (2DE), and gel images were compared by adequate software in order to establish characteristic protein signatures typical of Imatinib sensitive and Imatinib resistant cells. *Results.* Matrix assisted Laser Desorption Ionisation- Time of Flight Mass spectrometry (MALDI-TOF MS) analysis allowed the identification of 45 differentially expressed proteins We categorized these proteins into five main functional classes: i) Chaperones and Heat shock proteins ii) Nucleic acid interacting proteins (binding/synthesis/stability) , iii) structural proteins, iv) cell signalling and v) metabolic enzymes. i) Heat shock proteins HSP60 and HSP70isoform 1 and 2, valosin containing protein (VCP) known to bind the HSP90-interacting Bcr-Abl complex, resulted to be significantly over expressed in LAMA 84-R cells, indicating a possible involvement of several of these chaperone proteins in the mechanism of Imatinib resistance, via a possible block of bcr/abl proteosome degradation. ii) A relevant number of proteins interacting with DNA and RNA (hnRNPF, hnRNPH1, hnRNPK and eIF3) were found to be more abundant or even expressed only in imatinib resistant cells. iii) Structural proteins: vimentin, α tubulin, γ actin were instead significantly more expressed in imatinib sensitive cells. The identified proteins involved in cell signalling and in metabolic pathways (classes iv and v) resulted differentially expressed in LAMA 84-S and LAMA 84-R, but without a clear *signature*. *Summary/Conclusion.* Bcr/abl and FLT3 are client of chaperon protein HSP90 and it has been shown that HSP90 inhibitors are active in blocking CML cell proliferation. HSP70 is involved in inhibition of apoptosis. Thus the overexpression of these class of proteins seems to be directly responsible for the stability, maintenance and function of Bcr/abl, akt and other tyrosine kinase substrate of bcr/abl. This is fundamental for CML cells, basing imatinib resistance on constitutionally overexpression of bcr/abl. A similar pivotal role in the maintenance of the resistant phenotype may be attributed to RNA stabilizing proteins, like hnRNPF, hnRNPH1, hnRNPK. The optimal characterization of the protein signature of CML imatinib resistant cells and the identification of critical actors in drug resistance may lead to new therapeutic approaches, synergistic with all tyrosine kinase inhibitors at present in clinical trials. To our knowledge, this is the first direct proteomic comparison of imatinib sensitive versus resistant CML cells.

0150

DASATINIB IN PATIENTS WITH CHRONIC MYELOGENOUS LEUKEMIA IN MYELOID BLAST CRISIS (MBC) THAT IS IMATINIB-RESISTANT OR IM-INTOLERANT: RESULTS OF THE CA180006 START-B STUDY

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Background. Dasatinib (D) (BMS-354825) is an oral, multitargeted tyrosine kinase inhibitor of Bcr-Abl and SRC with activity against imatinib-resistant cell lines. A phase I study demonstrated preliminary evidence of activity of D in MBC-CML pts. *Aims.* To demonstrate the activity of D in pts who are resistant to or intolerant of imatinib. *Methods.* START B is an open-label study of D in IM-R or IM-I MBC carried out in 46 sites worldwide. From December 2004 to July 2005, 109 MBC pts were treated. D was given orally at 70 mg twice a day (BID) with dose escalation to 100 mg BID for poor initial response or dose reductions to 50 mg and 40 mg BID for toxicity. Pts had weekly blood counts and monthly bone marrow evaluations, including cytogenetics. Molecular monitoring of BCR-ABL transcript levels by RT-PCR were obtained at baseline and in pts who achieved cytogenetic response. The primary endpoint was confirmed (minimum 4 weeks duration) major hematologic response (MaHR). *Results.* Among the 109 treated patients, 99 were IM-R and 10 IM-I; median age was 55 yrs (range 21'81), 58% were male. Prior therapy included interferon in 53 (49%) pts, stem cell transplant in 15 (14%) pts and prior chemotherapy 49 (66%) pts. Prior IM dose was >600 mg/day in 49% of pts and 41% of pts received IM for > 3 years. WBC count $\geq 20\times10^3/mm^3$ in 46% of pts, Platelets count $< 100\times10^3/mm^3$ in 64% and 32% had ≥50% bone marrow blasts. Preliminary safety and efficacy analyses are currently available on the first 74 pts (68 IM-R, 6 IM-I). Mutations in the BCR-ABL domain were found in 27/63 (43%) pts. Median duration of therapy was 3.5 months. D doses were reduced in 35% of pts, temporarily interrupted in 58% pts, and escalated in 41% pts. With a minimum of 6 months follow-up, hematologic responses were seen in 39 (53%) pts: confirmed MaHR in 24 (32%) pts, Complete in 18 (24%) and No Evidence of Leukemia in 6 (8%). Major cytogenetic responses were documented in 22 (30%) pts and were complete in 20 (27%). The median time to MaHR was 56 days. None of the 24 pts who achieved a MaHR have relapse with a duration of MaHR ranging from 1.2+ to 7.8+ months. The median PFS had not been reached. Severe myelosuppression was common, but manageable. Non-hematologic toxicities were usually mild to moderate. The most common Grade 3-4 toxicities included diarrhea in (7%), pleural effusion (9%), nausea (4%). Peripheral edema was reported in 14% of pts (0% Grade 3-4), and rash in 11% of the pts (0% Grade 3-4). *Conclusion.* Dasatinib is highly effective in pts with IM-R MBC with durable MaHR. Data on all 109 pts will be presented at the meeting and will include the molecular response analysis.

0151

A PHASE II STUDY OF NILOTINIB (AMN107), A NOVEL INHIBITOR OF BCR-ABL, ADMINISTERED TO IMATINIB-RESISTANT OR INTOLERANT PATIENTS WITH PH+ CHRONIC MYELOGENOUS LEUKEMIA IN BLAST CRISIS OR RELAPSED/REFRACTORY PH+ ACUTE LYMPHOBLASTIC LEUKEMIA

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Background. Nilotinib is a highly selective, aminopyrimidine which *in vitro* is 30-fold more potent than imatinib, and active against 32/33 imatinib resistant Bcr-Abl mutations. *Aim.* This study was designed to evaluate the safety and efficacy of nilotinib as defined by hematologic/cytogenetic response (HR/CyR) rates in imatinib-resistant or intolerant patients, BC, or relapsed/refractory ALL patients. *Methods.* This is a Phase II, open-label, multicenter, study of nilotinib administered orally at a dose of 400 mg twice daily. *Results.* Preliminary data from this ongoing study are presented for 18 BC and 6 ALL (5 relapsed/refractory, 1 minimal residual disease) patients. Baseline mutation data are available for 6 BC and 2 ALL patients: 2 and 1, respectively, had Bcr-Abl mutations. The overall median age was 54 years and the overall median exposure was 84 days. Seven patients remain on treatment (5 BC, 2 ALL) and 17 discontinued (3 adverse events, 9 progressive disease, 3 deaths, and 2 other). Two BC patients died of disease progression. One Ph+ ALL patient had a sudden cardiac death. HR was reported in 7 (38%) BC patients; 5 complete HR and 2 marrow responses/no evidence of leukemia. Three BC patients had CyR (2 complete, 1 minor). Complete remissions were reported in 2 (33%) ALL (1 relapsed/refractory and 1 MRD) patients. Adverse events occurring in ≥ 10% of patients were mostly grades 1 or 2 and encompassed rash 37% (n=9), thrombocytopenia 33% (n=8), nausea, pyrexia, vomiting 29% (n=7 each), diarrhea, fatigue, headache 25% (n=6 each), anemia, extremity pain 21% (n=5 each), peripheral edema, pruritis 17% (n=4 each), and leukocytosis, arthralgia, pharyngolaryngeal pain, upper abdominal pain in 13% (n=3 each). Overall Grade 3/4 adverse events included thrombocytopenia 29% (n=7), neutropenia 25% (n=6), anemia 17% (n=4), pyrexia 8% (n=2), and leukocytosis, pruritis 4% (n=1 each). *Summary/Conclusions.* Nilotinib has an acceptable safety and tolerability profile and is active in patients with imatinib-resistant or intolerant BC and relapsed/refractory Ph+ ALL.

0152

A STUDY OF BCR-ABL MUTATIONS IN PATIENTS WITH IMATINIB-RESISTANT CML AND PH+ALL PATIENTS ENROLLED IN A NILOTINIB (AMN107) PHASE I STUDY

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Background. Nilotinib is a potent, highly selective, aminopyrimidine inhibitor which *in vitro* is 30-fold more potent than imatinib and active against 32/33 imatinib resistant Bcr-Abl mutations. *Aim.* To evaluate the spectrum of mutations in Bcr-Abl was evaluated by direct sequencing of the kinase domain and surrounding regions on samples from patients with imatinib-resistant Ph+ CML or ALL. *Methods.* This is a Phase I study of nilotinib (total oral doses ranging from 50 to 1200 mg administered daily or twice daily). The template was created by semi-nested PCR using primers in the BCR and ABL regions of the gene. Mutations correlated with clinical response. *Results.* There were 119 patients enrolled of whom 86 had both pre and post treatment analyses. Mutations were present at baseline for 39 (45%) patients of whom 27 (69%) responded. Of the 86 patients with data available 47 (55%) had no mutation at baseline of which 34 (72%) responded. The most common baseline mutations were G250E, E255K, E355G, F317L, H396R and M351T. New mutations were found during median follow-up of 112 (6 - 350) days in 37 patients (26 without baseline mutations). New most commonly included F359V, E255K/V, E355G, G250E, M244V and T315I. Of the 37 patients, 30 had evaluations after mutations emerged, and 15 continued to respond for median of 160 (41-351) days. Fourteen mutations not previously reported occurred, 6 at codons with known imatinib-resistance mutations resulting in novel amino acid substitutions. New mutations in several patients included E334G, F311I, E453K, and E459Q. The T315I mutation was present at baseline in 1 patient who failed to respond, and emerged in 4 patients, of whom follow-up was available for three. Two continued to respond ≥ 80 days after developing the mutation and one progressed when the mutation emerged. *Summary/Conclusions.* Nilotinib has clinical activity in patients with Ph+ CML/ALL with nonmutated and mutated Bcr-Abl. At nilotinib doses used in this Phase I study, new mutations often identified during follow-up, but were not a reliable predictor of clinical relapse. Future studies using more sensitive methods of mutation detection, such as D-HPLC, are needed to determine whether mutations detected during AMN107 therapy are present at low levels prior to therapy.

0153

ROLE OF ENDOTHELIAL PROGENITOR CELLS IN MYELOPROLIFERATIVE DISEASES

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Background. The presence of circulating hematopoietic progenitor cells has been described in patients with myeloproliferative diseases (MPD). However, the exact nature of such progenitor cells has not been specified until now. *Aim.* The aim of this work is to proof the hypothesis that the endothelial cell lineage is primarily involved in the pathophysiology of myeloproliferative diseases. *Methods.* Expression of the hemangioblast markers (early common precursors to the hematopoietic and endothelial cell lineage) in the circulating cells of 53 patients with MPD was assessed. Peripheral blood was analysed for expression of CD34, prominin (CD133), KDR (kinase insert domain receptor, or vascular endothelial growth factor receptor 2, VEGFR2) and vWF (von Willebrand factor) mRNA by quantitative PCR. Clonogenic stem cell assays were performed to assess differentiation towards the hematopoietic and endothelial cell lineage. Patient data (essential thrombocythemia (ET, n=17), polycythemia vera (PV, n=21) and chronic idiopathic myelofibrosis (CIMF, n=15)) were compared with data from normal controls (n=16) and patients with secondary thrombo- or erythrocytosis (n=17). *Results.* Trafficking of CD34 positive cells was increased above the physiological level in 4/17 patients with ET, 5/21 patients with PV and 13/15 patients with CIMF. A subset of patients with CIMF co-expressed the hemangioblast markers CD34, Prominin (CD133) and KDR, suggesting the presence of hemangioblasts among the circulating progenitor cells. Clonogenic stem cell assays confirmed differentiation towards both the hematopoietic and the endothelial cell lineage in 5/10 patients with CIMF. Furthermore, trisomy 8 was found in the grown endothelial cells of a patient in which trisomy 8 was diagnosed in the peripheral blood, confirming the common clonal origin of both cell lineages. *Conclusion.* Hemangioblasts are present in the blood of a subset of patients with CIMF, suggesting a primary role of pathological endothelial cells in this disease.

0154

COMPLIANCE AND PERSISTENCY WITH IMATINIB IN CHRONIC MYELOID LEUKEMIA PATIENTS

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Background. Imatinib is an oral molecularly targeted therapy with unprecedented efficacy in chronic myelogenous leukemia (CML) and gastrointestinal stromal tumors (GIST). Optimal dosing and adherence to treatment is critical to achieve the best clinical outcomes. *Aims.* This study examined compliance and persistency with imatinib in CML patients and identified the clinical and patient characteristics that are related to compliance and persistency. *Methods.* Claims data from a large US health plan were used to identify imatinib-treated patients from 6/1/01-3/31/04 who had continuous pharmacy and medical benefits in the 3 months prior and 12 months following initiation of imatinib therapy, and a diagnosis of CML (ICD-9-CM 205.1, 205.10, or 205.11). Compliance was defined by medication possession ratio (MPR=total days supply of imatinib in the first year divided by 365). Persistency was defined as failure to refill imatinib within 30 days from the run-out date of the prior prescription. Multivariate analyses were used to identify the key factors that are associated with compliance and persistency. *Results.* Total 878 imatinib-treated patients were identified of whom 413 had at least 15 months' continuous eligibility. Sixty-nine percent (n=286) were diagnosed with CML and are the subjects of this analysis. The average age was 50 (range from 3 to 86, median 50.5) and 58% were males. The average starting daily dose was 423 mg, with 81% (n=232) initiating on 400 mg daily. The mean MPR was 76%. Overall, 32% patients discontinued imatinib for at least 30 consecutive days during the 1-year follow up period. Multivariate analyses indicated MPR improved with age until age 52 and then deteriorated ($p<0.001$) but at a diminishing rate, decreased as the number of other medications used by the patient increased ($p=0.01$), and was lower in women ($p=0.004$) and patients with more cancer complications ($p=0.005$). Other variables included in this analysis were starting daily dose and geographic region. In the multivariate analysis of the likelihood to discontinue imatinib for at least 30 consecutive days, women were found to be more likely to discontinue than men (OR=2.33; $p=0.003$) controlling for age, starting dose, cancer severity, and number of other medications used by the patient. *Conclusions.* Compliance to imatinib was about 75% with over 30% of patients interrupting therapy for at least 30 consecutive days in the first year. The MPR of 76% in this population appears to be lower than the reported average of 97% relative dose intensity in clinical trials of imatinib (including high-dose imatinib). This suggests there may be less compliance in patients not enrolled in clinical trials. It has been found that interruption of imatinib therapy in CML may lead to rapid relapse or recurrence of disease (Cortes *et al.* Blood 2004; Mauro *et al.* Leukemia Research 2004). Since compliance may affect clinical outcome, physicians should educate patients and closely monitor compliance to therapy.

0155

COMPARISON BETWEEN CONVENTIONAL AND MOLECULARLY DEFINED THERAPEUTICS IN A CONDITIONAL BCR-ABL CELL CULTURE MODEL

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Background. Chronic myelogenous leukemia (CML) is a myeloproliferative disease in which the constitutively active tyrosine kinase BCR-ABL enhances survival of leukemic cells through modulation of intracellular signaling cascades. Accordingly, current therapeutic strategies include ABL-specific tyrosine kinase inhibitors like Imatinib (IM). However, recent studies have demonstrated drug resistance and persistance of leukemic (stem) cells under IM therapy. Therefore, combination therapy directed to a complementing target may significantly improve treatment *Results.* We recently identified such potential targets by demonstrating that RNAi mediated reduction of SHP2, STAT5, and Gab2 protein expression inhibits BCR-ABL but not cytokine dependent proliferation (Blood. 2005 Nov 8; [Epub ahead of print]) *Aims.* In the current study we compared the specificity and efficacy of molecularly defined therapeutics such as IM or shRNAs inhibiting expression of SHP2, STAT5, and GAB2 with that of conventional antileukemic drugs in a conditional BCR-ABL cell culture model. *Methods.* We used the TonB cell line which is IL3 dependent but can be induced to express BCR-ABL by doxycycline, resulting in cytokine independent proliferation. To specifically silence expression of SHP2, STAT5 and GAB2, TonB cells were transduced with Pol III driven expression cassettes for specific shRNA transcription by lentiviral gene transfer. In addition lentiviral transgene plasmids driving the simultaneuos expression of two shRNAs have been generated. Cytarabine, doxorubicin, and etoposide were used as non-specific conventional drugs. *Results.* We already demonstated that inhibition of SHP2, STAT5, and Gab2 expression by RNAi specifically inhibits BCR-ABL mediated proliferation in TonB cells (Blood, in press). In contrast to shRNAs, we demonstrate here that cytarabine, etoposide, and doxorubicin inhibit both IL3 and BCR-ABL mediated cell proliferation in TonB cells. Based on the number of viable cells we determined very similar LD50 values for cytarabine (300 ng/mL, 220 ng/mL), etoposide (150 ng/mL 110 ng/mL) and doxorubicin (4 ng/mL, 3.8 ng/mL) for TonB cells grown in the presence of IL3 or BCR-ABL, respectively. Next we combined cytarabine (300 ng/mL), etoposide (150 ng/mL) and doxorubicin (4 ng/mL) with IM. Cytotoxic drugs cooperate with IM, with complete loss of viable TonB cells in BCR-ABL mediated cell proliferation, but also 50% cell death in IL3 mediated cell proliferation. The combination treatment with shRNAs against SHP2, STAT5, and GAB2 with antileukemic drugs also resulted in more than 95% cell death in BCR-ABL mediated cell proliferation and 50-70% cell death in IL3 mediated cell proliferation. The combination of two shRNAs such as anti-BCR-ABL-anti-SHP2 and anti-BCR-ABL-anti-STAT5 or the simultaneous application of IM with anti-SHP2, anti-STAT5, and anti-GAB2 resulted in complete cell death in BCR-ABL mediated cell proliferation but no inhibition in the presence of IL-3. *Conclusion.* Conventional antileukemic drugs have no differential effects on BCR-ABL and IL-3 mediated cell proliferation whereas targeting of SHP2, GAB2, and STAT5 by shRNAs specifically inhibits oncogene driven proliferation of TonB cells. Combined therapy with molecularly defined therapeutics e.g. imatinib mesylate with anti-SHP2, anti-STAT5, anti-GAB2 shRNA or two shRNAs (anti-BCR-ABL-anti-SHP2 and anti-BCR-ABL-anti-STAT5) cooperates in inhibiting BCR-ABL driven TonB cell proliferation with no or only minor effects on IL-3 dependent growth. Further studies on primary cells are warranted to further analyze the specificity and efficacy of combined targeted therapies.

0156

A PHASE II STUDY OF NILOTINIB (AMN107) A NOVEL INHIBITOR OF BCR-ABL, ADMINISTERED TO IMATINIB-RESISTANT OR INTOLERANT PATIENTS WITH CHRONIC MYELOGENOUS LEUKEMIA IN ACCELERATED PHASE

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Background. Nilotinib is a potent, highly selective, aminopyrimidine inhibitor of Bcr-Abl which *in vitro* is 30-fold more potent than imatinib. It is active against 32/33 imatinib resistant Bcr-Abl mutations. *Aim.*This study was designed to evaluate the safety and efficacy of nilotinib as defined by hematologic and cytogenetic response (HR/CyR) rates in imatinib-resistant or intolerant AP patients. *Methods.* This is a Phase II open-label, multicenter, study of nilotinib administered orally at a dose of 400 mg twice daily. *Results.* This study remains open to enrollment. Preliminary data are reported for 22 patients (77% resistant and 23% intolerant to imatinib). The median age was 62 (range 43-76) years and the median time from AP diagnosis was 6 months (range 0.2-56). Three of five patients with data available had a BCR-ABL mutation at baseline. The median duration of nilotinib exposure was 124 days (range 3-207). Treatment is ongoing for 16/22 (73%) patients. HR occurred in 14 (64%) patients of which 10 (45%) were complete. Three (14%) were marrow responses/no evidence of leukemia, and 1 had a return to chronic phase. CyR occurred in 6 patients (1 complete, 1 partial, 1 minor, and 3 minimal). The AE's occurring in ≥ 10% patients were thrombocytopenia 36% (n=8), fatigue 32% (n=7), anemia, pruritis, muscle spasms 27% (n=6 each), bone pain, cough, rash 23% (n=5 each), diarrhea, headache, myalgia, pyrexia 18% (n=4 each), abdominal pain, chills, constipation, dyspnea, nausea, extremity pain, and peripheral edema 14% (n=3 each). The overall incidence of Grade 3/4 AE's were thrombocytopenia 27% (n=6), anemia, neutropenia 18% (n=4 each) and rash 5% (n=1). Two deaths occurred one patient with thrombocytopenia had a CNS bleed and one patient had disease progression. *Summary/Conclusions.* These data suggest that nilotinib is clinically active and has an acceptable safety profile when administered to patients with CML-AP.

0157

IMATINIB 800 MG IN INTERMEDIATE SOKAL RISK PATIENTS IN EARLY CHRONIC PHASE: RESULTS OF A PHASE II TRIAL OF THE GIMEMA CML WORKING PARTY

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Background. Imatinib standard dose (400 mg) gives impressive results in chronic myeloid leukemia (CML) in early chronic phase. Results, stratified by Sokal risk, are inferior in intermediate and high risk with respect to low risk.In intermediate Sokal risk patients, the IRIS trial (T Hughes *et al.,* NEJM 349:15, 2003) reported within 12 months a complete cytogenetic response (CCgR) rate of 67% and a major molecular response (MMR) rate of 45%. Phase I and II trials of imatinib have clearly shown a dose response effect. Kantarjian *et al.,* (Blood 103, 2004) reported higher response rates with imatinib high dose (800 mg) in 114 early chronic phase patients treated at the MD Anderson Hospital. The CCgR was 90% and MMR 60% within 12 months of therapy. *Aims.* The GIMEMA CML WP opened in January, 2004 a phase II, multicentric prospective study (serial n. CML/021) devoted to investigate the effect of imatinib high dose (800 mg) in intermediate Sokal risk patients. *Methods.* Clinical and anagraphical data were collected through a web-based system. Responses were evaluated at fixed time-points during treatment. Hematologic: continuosly; cytogenetic at 6 and 12 months (local labs); molecular response at 3, 6 and 12 months.Peripheral blood samples for quantitative molecular analysis (RT-Q-PCR, Bcr-Abl/Abl × 100 - Taqman) were centralized in Bologna at 3, 6 and 12 months. *Patients*. Between January 1, 2004 and April, 2005 23 italian centers enrolled 81 patients (72 evaluable). Median age was 56 yrs (range 26-79), 43 males and 29 females. 72 patients are evaluable for response at 3 months, 65 at 6 months and 43 at 12 months. The median observation time is 6 months. *Results.* At 3 and 6 months, 81% and 98% of the patients reached a stable complete hematologic response, respectively. At 6 months, 87% of the evaluable cases obtained a CCgR (100% Ph-neg). A MMR defined as a Bcr-Abl/Abl x 100 ratio < 0.1%, was shown in 53% of CCgR patients. At 12 months, the CCgR rate was 90% and the MMR rate in CCgR patients was 56%. The cumulative incidence of CCgR was 94%. 2 pts progressed to accelerated/blastic phase. 56%, 51% and 53% of the pts received 100% of the scheduled dose at 3, 6 and 12 months. *Summary and Conclusions.* The preliminary results of our trial suggest that imatinib 800 mg is highly effective for intermediate Sokal risk CML in early chronic phase, being superior to 400 mg (IRIS trial, same risk category) and in the range of the MD Anderson results.

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0158

PHILADELPHIA-POSITIVE HEMATOPOIETIC PROGENITORS ARE NOT DETECTABLE IN CML PATIENTS TREATED WITH IMATINIB ACHIEVING A MAJOR MOLECULAR RESPONSE

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Background. Imatinib mesylate can remarkably diminish the leukemic burden and produce complete cytogenetic response (CCR) in the majority of patients and some degree of molecular response. However, the clearance of the vast majority of Ph-positive cells does not necessarily translates in the eradication of the disease. The leukaemic stem cells are operationally encompassed within the CD34+ cells and CD34 derived progenitors (CFC, LTC-IC). In this study, we investigated the number of residual BCR-ABL+ hematopoietic progenitors on BM samples from CML pts who reached a CCR with Imatinib. *Materials and Methods.* BM samples were obtained from 22 CML pts in CCR under Imatinib. All of them were in chronic phase. CD34+ cells were selected using a double immunomagnetic column separation (Miltenyi Biotech). CFCs and LTC-ICs were obtained by seeding the CD34+ selected population using standard culture *Methods.* FISH studies for the BCR-ABL fusion gene (Dual color, Dual Fusion DNA probe, Vysis, Downer Grove) were performed on freshly selected CD34+ cells, CFCs and LTC-ICs. A total of 100-200 nuclei were scored for each sample. The normal cut-off value used for these probes was less than 1%. Major Molecular Response (MMR) was defined as BCR-ABL/ABL ratio less than 0.05% (roughly equivalent to a 3-log disease reduction). Real-time quantitative RT-PCR was performed using a TAQ-Man system for BCR-ABL and ABL genes. The median follow-up for pts in MMR or not in MMR was 34 (14-54) and 36 (6-59) months, respectively. *Results.* The purity of selected CD34+ cells was 95% (82-99). MMR was present in 14/22 (55.5%) CML pts. In patients not in MMR (n=8) residual percentage BCR-ABL+ cells was detected on CD34+ (3-8%), CD34+ cells derived CFCs (3-7%) and LTC-ICs (3-5%). None of the patients in MMR (n=14) showed residual BCR-ABL+ progenitor cells: selected CD34+ cells CD34+ cells derived CFCs and LTC-ICs were 100% Ph-negative; (MMR vs not MMR $p<0.0001$ by Fisher's test). *Conclusions.* Previous reports found that CML pts in CCR under Imatinib have residual BCR-ABL+ hematopoietic progenitors. Our data indicate that the detection of leukemic progenitors differs significantly between molecular responders and non-responders, thus suggesting a correlation between QRT-PCR and of the number of residual leukaemic progenitors. However, since the average frequency of Ph-positive early progenitors is considerably low in CML patients at diagnosis (more than 70-80% of LTC-IC being Ph-negative) these data suggest that in patients without molecular response the impact of Imatinib on the early progenitor pool may be limited. Further studies are necessary to identify a better read-out of residual Ph-positive stem cells.

0159

DASATINIB (D) IN PATIENTS (PTS) WITH ACCELERATED PHASE CHRONIC MYELOID LEUKEMIA (AP-CML) RESISTANT OR INTOLERANT TO IMATINIB: RESULTS OF THE CA180005 'START-A' STUDY

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*Background.*D (BMS-354825) is an oral multi-targeted kinase inhibitor with preliminary evidence of efficacy in a previously reported phase I study. *Aims.* To demonstrate the activity of D in patients (pts) with AP-CML resistant to or intolerant of imatinib. *Methods.* START A is an open-label study of dasatinib in AP-CML who were imatinib resistant (IM-R) or imatinib intolerant (IM-I). Dasatinib was given orally at 70 mg twice daily (BID). Dose escalation to 100 mg BID was allowed for inadequate initial response and reduction to 50 or 40 mg BID for persistent toxicity. Evaluation included weekly blood counts and monthly bone marrow including cytogenetics. Molecular evaluation of Bcr-Abl transcript by real-time quantitative polymerase chain reaction was performed at baseline and after documentation of complete cytogenetic response. The primary endpoint was major confirmed (maintained at least 4 weeks) hematologic response (MaHR) in IM-R pts. *Results.* A total of 174 pts (161 IM-R and 13 IM-I) were enrolled between December 2004 and July 2005 in 39 centers worldwide. There were 96 (55%) males; median age 57 yrs (range 22-86); median time from diagnosis of CML 82 months. Prior therapy included IM>600 mg/day in 91 (52%) pts, IM for > 3 years in 103 (59%) pts, interferon in 126 (72%) pts, stem cell transplantation in 23 (13%) pts. Major Cytogenetic Response (MCyR) to prior IM was seen in 57 (33%) pts. Preliminary assessment of efficacy and safety was performed on the first 107 pts (99 IM-R, 8 IM-I) with ≥6 months of follow-up. The average daily dose was 119 mg/day; 52 (49%) pts required a dose reduction mostly due to hematologic toxicity. MaHR was documented in 63 (59%) pts (95% CI: 49-68) with Complete Hematologic Response in 35 (33%) and No Evidence of Leukemia in 28 (26%). MCyR was documented in 33 (32%) pts (95% CI: 22.9'41.6); complete in 23 (22%), partial in 10 (10%). In the 99 IM-R pts, the MaHR rate was 59%. In the 56 pts with Bcr-Abl mutations the MaHR was 66%. Molecular response analysis is ongoing. Fifteen pts had disease progression including one loss of MaHR. Myelosuppression was significant with grade 3'4 thrombocytopenia and neutropenia in 79% and 69% of pts, respectively. Non-hematologic toxicities were generally mild to moderate. The most frequent were diarrhea (46%), peripheral edema (27%), pleural effusion (16%), rash (8%), and GI hemorrhage (7%). *Conclusions.* Dasatinib is very effective in pts with IM-R AP-CML with high rates of durable MaHR and MCyR. Data on all 174 pts will be presented at the meeting including the molecular response analysis.

0160

INVOLVEMENT OF RAS, JAK2 AND GM-CSF IN THE PATHOGENESIS OF PROLIFERATIVE VARIANT OF CHRONIC MYELOMONOCYTIC LEUKEMIA

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Background. Chronic myelomonocytic leukemia (CMML) is a heterogeneous malignancy classified among MDS/MPD disorders. The paucity of known pathogenetic events contributes to the lack of effective treatment and to its dismal prognosis. On the basis of the peripheral leukocyte count, a *dysplastic* subtype (MD-CMML, WBC $<12\times10^9$/L) can be distinguished from a *proliferative* subtype (MP-CMML, $>12\times10^9$/L WBC/L) of the disease. Among factors that have been implicated in pathogenesis of CMML, GM-CSF produced by either autocrine or paracrine mechanisms has been shown to be a major growth determinant. *Aims.* To investigate cellular and molecular differences between MD- and MP-CMML which could contribute in clarifying pathogenesis of the disease and in identifying targets for possible therapeutic applications. *Methods.* Peripheral blood mononuclear cells (MNC) were isolated on Ficoll-Paque density gradient from 29 patients affected by CMML (17 with MD-CMML and 12 with MP-CMML). Samples were screened for the presence of N/K-RAS genes mutations by PCR and direct sequencing. Identification of the JAK2 V617F mutation was carried out by both allele-specific PCR and amplification of exon 12 followed by restriction analysis. Also, to evaluate the expression of intracytoplasmic GM-CSF and the expression of its receptor (GM-CSFR), MNC were stained with GM-CSF PE (Caltag) and CD116 (Pharmingen), respectively. *in vitro* growth of myeloid colonies was assessed in semisolid medium with or without the addiction of cytokines (SCF, GM-CSF, IL-3, Epo). *Results.* No RAS or JAK2 mutations were detected in the group of patients with MD-CMML. In the proliferative variant group, we identified two patients carrying the G12D substitution of N-RAS. Furthermore, a G60E point mutation of N-RAS was identified in 1 patient after progression from MD- to MP-CMML. The JAK2 V617F mutation was detected in 4 patients, all affected by the proliferative variant of CMML. Mean percentage of GM-CSF expression was 59.8 (range 14.5-90.7) in MP-CMML and 2.27 (range 0-9.3) in MD-CMML. The difference between MP and MD disease was statistically significant. In contrast, mean percentage of expression of GM-CSFR was similar in MD- and MP-CMML samples (40,1 vs 42,2). However, when we considered median intensity of the GM-CSFR expression, we observed significantly higher values in MP-CMML than in MD-CMML (123.2 and 51.4, respectively). The number of CFU-GM was higher in the MP-CMML than in MD-CMML (57 vs $17/5\times10^5$/L cells plated) and a significant correlation with intracytoplasmic GM-CSF expression was observed ($p<0.05$). *Conclusions.* In summary, in our series of patients with proliferative variant of CMML, RAS and JAK2 mutations were quite frequent (25% and 33%, respectively). Because MP-CMML may evolve from MD-CMML, these findings support the hypothesis that molecular abnormalities could be acquired with disease progression. Moreover, since both JAK2 and RAS proteins are involved in the GM-CSF signalling pathway, the higher levels of intracytoplasmic cytokine and the increased density of its receptor in MP-CMML support the hypothesis that this pathway has a central role in malignant cell proliferation of CMML patients.

Chronic myeloid leukemia II

0161

TRANSCRIPTIONAL PROFILING OF PRIMARY IMATINIB-RESISTANT CHRONIC MYELOID LEUKEMIA

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Background. Although the selective tyrosine kinase inhibitor imatinib is successfully used in the treatment of chronic myeloid leukemia (CML), inherent mechanisms confer primary resistance in a significant minority of patients. *Aims.* In order to search for potentially useful genes in predicting cytogenetic response, a retrospective microarray-based gene expression study was performed. *Methods.* Quality-controlled RNA from leukocytes of bone marrow (BM) and/or peripheral blood (PB) of 34 interferon-α-pretreated chronic phase CML patients with or without major cytogenetic remission (£ 35% Ph+ metaphases) during the first year of imatinib treatment was comparatively analyzed using high-density Affymetrix U133A chips. Diagnostic groups (responders, n=23; non-responders, n=11) were matched according to demographic and hematologic parameters and evaluated during their clinical course for hematologic, cytogenetic and molecular responses. For the assessment of differences in gene expression, BM and PB samples simultaneously taken from seven imatinib responders were statistically analyzed based on mixed loglinear models. *Results.* Using support vector machines for gene classification, an outcome-specific gene expression signature consisting of 128 genes was identified. Comparative expression data of specific genes point to changes in apoptosis (e.g. casp9, trap1, hras), DNA repair (msh3, ddb2), oxidative stress protection (gss, pon2, vnn1), and centrosomes (id1) within primary resistant patients. Independent statistical approaches (ANOVA, PAM) as well as quantitative real-time PCR studies on a selected subset of genes (vnn1, rph3a, tpsab1/b2, coch) verified the validity of the obtained data. Furthermore, the potential 128-gene predictor was tested on two independent patients with primary resistance that became accessible after having completed the study. Both test set patients were correctly assigned to their respective diagnostic group. Prospectively, our candidate predictor will be further explored on samples from CML patients currently treated in clinical studies. *Conclusions.* This study establishes a candidate 128-gene predictor for early assessment of primary cytogenetic response of CML patients to imatinib. The data suggest that transcriptional regulation of genes related to apoptosis, disease progression, oxidative stress, DNA repair and centrosome fidelity are associated with imatinib resistance in chronic phase CML.

0162

MONITORING SERUM LEVELS OF IMATINIB AND HAEMATOLOGICAL TOXICITY IN CML PATIENTS

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Background. The development of imatinib mesylate (Glivec®) has been a major advance in the management of chronic myelogenous leukaemia (CML). Although several clinical trials, revealed neutropenia, thrombocytopenia and/or anaemia in a proportion of patients on imatinib treatment, little is known about the potential association of these adverse effects and the serum levels of imatinib. *Aims.* We have developed a new method to analyze serum concentrations of imatinib mesylate and its active metabolite, N-demethyl-imatinib in Ph+CML patients. The aim of our study is to determine whether there is an association between serum levels of the drug and the development of either anaemia, neutropenia or thrombocytopenia, in order to establish an optimal therapeutic range. *Methods.* We used a Beckmann Gold HPLC system in reverse phase under isocratic conditions, coupled to a visible ultraviolet detector fixed at 240 nm wavelength. We analyzed serum concentrations of imatinib and N-demethyl-imatinib in 114 samples from 36 patients with Ph+CML at different intervals. There were 19 males and 17 females, with a mean age of 57±15 years. Each patient was followed for at least six months. *Results.* Serum levels of imatinib showed a wide variation ranging from 0.43 µg/mLto 5.19 µg/mL. There was statistical correlation between therapeutical dose of Glivec® and serum concentration of the drug (r=0.47). Anaemia was present in 21 cases (58.3%), neutropenia in 3 cases (8.3%) and thrombocytopenia in 6 patients (16.5%). Patients with anaemia showed a mean concentration of imatinib of 2.10 µg/mL, compared to 1.41 µg/mL in non anaemic patients (p=0.015). Significantly higher levels of imatinib were also observed in patients with thrombocytopenia (2.60 µg/mL vs 1.70 µg/mL, p=0.047). No significant differences were observed with respect to neutropenia. Serum concentrations of imatinib above 3 µg/mL were strongly associated to adverse events requiring transient discontinuation of the drug (p<0.001). *Conclusions*. The development of either anaemia or thrombocytopenia in CML patients on imatinib therapy is associated with high serum concentrations of this drug. When serum levels of imatinib rise above 3 µg/ml, the incidence of adverse events is high, and, therefore, the dose should probably be reduced. Taken together, our results demonstrate the feasibility and utility of monitoring serum levels of imatinib in CML patients. An optimal therapeutic range of between 0.57 µg/mL and 2 µg/mL is suggested.

0163

IMATINIB PRECEDING ALLOGENEIC STEM CELL TRANSPLANTATION IN CHRONIC MYELOID LEUKAEMIA

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Background. Little is known on Imatinib treatment prior to allogeneic stem cell transplantation in chronic myeloid leukaemia (CML). *Aim.*In order to investigate the outcome of allogeneic stem cell transplantation after Imatinib prior to conditioning we analysed retrospectively the engraftment rate, incidence of acute and chronic graft-versus-host disease (aGvHD and cGvHD) and transplant related mortality (TRM) in adult patients (pts.) with chronic myeloid leukaemia (CML), who received peripheral blood stem cell (PBSCT) or bone marrow transplantation (BMT) from sibling (SIB) or volunteer unrelated donors (VUD). *Patients and Methods.* 37 pts. (23 male, 14 female; median age 31 y, range 16-55) were treated with SIB (n=6) or VUD (n=31) allogeneic SCT (n=11) or BMT (n=26) for CML in first chronic (CP1, n=13) or accelerated phase (AP, n=17)or for more advanced disease stages: second chronic phase or blast crisis (CP2 or BC, n=7). Transplant conditioning consisted of fractionated total body irradiation (TBI) and cyclophosphamide (120 mg/kg) in all pts. All but one recipients of VUD transplants received *in vivo* T-cell depletion (TCD) with CAMPATH 1H (Anti CD52, n=29) or ATG (n=1). In all pts. GvHD prophylaxis was provided by Cyclosporin A and short-term methotrexate. *Results.* 7 pts. received Imatinib for treatment of BC, 30 pts. with the aim to achieve haematological or cytogentic remission in CP1 or AP. The median duration of Imatinib pre-treatment was 8.5 months (range 1-31 months). The used drug dose ranged from 200mg to 800mg. Imatinib was discontinued within 8 weeks prior transplant in 26 pts. 11 pts. had a longer than 8 weeks break between Imatinib and SCT. Engraftment failure occurred in 1 patient (2.7%). The probability of TRM at day 100 was 13%, which increased to 38% at 1 year. aGvHD grade III-IV occured in 8 pts (22%), whilst extensive cGvHD was seen in 10 pts (31%). The overall survival is 65% after a median follow up of 203 days (14-1419 days). *Conclusion.* In 37 pts. treated with Imatinib prior to allogeneic SCT or BMT the overall rates for engraftment failure, TRM, aGvHD and cGvHD were comparable with previously published data in BMT or SCT in CML without Imatinib pre-treatment and with our institution's experience prior to the Imatinib era. Imatinib prior to allogeneic transplantation does not seem to have a negative impact on the tranplantation outcome. Further investigations on larger cohorts are needed in this field.

0164

EARLY OR LATE COMPLETE MOLECULAR REMISSIONS IN CHRONIC MYELOGENOUS LEUKEMIA PATIENTS TREATED WITH IMATINIB RELY ON THE SPEED TO ACHIEVE A COMPLETE CYTOGENETIC REMISSION STATUS AND ON EARLY QUANTITATIVE BCR-ABL LEVELS

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In chronic phase (CP) chronic myelogenous leukemia (CML) treated with imatinib (IM), the majority of patients that are in CCR still harbour, apparently indefinitely, a stable molecular disease, while IM is maintained. A small proportion of patients become RQ-PCR negative (transcripts levels <10-5) and there is still some controversy to know if these patients are cured. We retrospectively analyzed a cohort of 37 patients that became, at least once, BCR-ABL negative in the blood (Taqman™ technology, sensitivity threshold 10-5-10-6, exchange of quality control samples between the 2 laboratories involved), to try to identify predictive factors for early [<12 Months, Group 1 (G1), 19 patients] versus late [>12 Months, Group 2 (G2), 18 patients] complete molecular remissions (CMR), in an univariate and multivariate analysis. Allogeneic transplanted patients were excluded form this study. All patients were in CP at diagnosis except 2 [1 in accelerated phase diagnosed on cytological criterias (G1), and 1 in myeloid blastic phase (G2)]. All patients had a 'M'-BCR transcript, and were 90-100% Ph1+ at diagnosis, except 1 [61% Ph1+ G1)]. One patient had a masked Ph1 and 1 had a variant [t(2;9;22), (G1)]. There were 19 males and 18 females with a median age of 52 (G1) and 50 (G2). Some patients have been treated with IFN prior to IM (12/19 in G1 and 13/18 in G2). All patients received IM 300-600 mg/day and some of them in association with Cytarabine (2 in G1; 2 in G2), PegIFN (1 in G1; 1 in G2), or daunorubicine + cytarabine (Patient in blastic phase: G2). One patient was in CMR, after IFN, when IM was started for IFN intolerance. None of the patients was in CMR at 3 Mo except 2 in G1. CMR appeared after a median interval of 6 Mo in G1 (0-11.2) and 25 Mo in G2 (12-53.7). Univariate analysis did not found any difference for prior treatment by IFN, Sokal score and associations with Cytarabine or PegIFN. Analysis of variance indicated that a low RQ-PCR value at 6 Mo and at 12 Mo was a significant factor for early CMR (p=0.03 for both). Mulivariate analysis by logistic regression for Sokal score, prior treatment with IFN, initial IM dose had no influence on the early or late CMR status, whereas the interval between diagnosis and IM onset (p=0.04, HR=1.45 [1.01-2.08]), probably because there was a longer exposure to IFN; and a shorter time to reach a CCR status were significantly associated with early CMR (p=0.03, HR=0.67 0.46-0.97]). However, the duration of IFN treatment variable was not significant by itself. With a median follow-up since IM onset of 24 (G1) and 43 (G2) Mo, all patients are alive, 16 out of 19 in G1 and 15 out of 18 in G2, in a stable CMR. In conclusion, when IM induces early CCR, and a quick reduction of BCR-ABL transcripts initially, CMR can be obtained within a year, but CMR are still possible after a longer period. However gain in progression free survival remains to be demonstrated.

0165

THE IMPACT OF NON-COMPLIANCE WITH IMATINIB THERAPY ON HEALTH CARE COSTS IN CHRONIC MYELOID LEUKEMIA PATIENTS

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Background. While compliance to drug therapy is vital to receive optimal patient benefits, the costs of delivering adequate medical care for cancer patients remain an important consideration for society and payers. *Aims.* This study examined the relationship between compliance with imatinib therapy and health care costs for patients with chronic myeloid leukemia (CML). *Methods.* Claims data from a large US health plan were used to identify imatinib-treated patients from 6/1/01-3/31/04 who had continuous pharmacy and medical benefits in the 3 months prior and 12 months following initiation of imatinib therapy, and a diagnosis of CML (ICD-9-CM 205.1, 205.10, or 205.11). Compliance was defined by medication possession ratio (MPR=total days imatinib supply in the first year divided by 365) and patients were stratified into three segments by MPR (<50%, 50-90%, 90-100%). Total health care costs include hospital, office, laboratory testing, emergency room, and pharmacy. Disease-related health care costs to treat CML were also analyzed. Multivariate analyses were used to examine the association between first-year MPR and first-year health care costs, controlling for age, sex, number of other medications used by the patient, initial starting dose, year of initial imatinib fill, and complications due to underlying disease. *Results.* Total 878 imatinib-treated patients were identified of whom 413 had at least 15 months of continuous eligibility. Of these, 277 were non-Medicare CML patients. Total health care costs per patient in the first year of therapy in patients with MPR < 50%, 50-90%, and 90-100% were $172777, $54479, and $41391 respectively ($p$<0.001). Inpatient care was the leading driver of health care costs followed by medication use and ambulatory care. The corresponding numbers for disease-related health care costs were $112905, $38561, and $34964 ($p$<0.001). Controlling for the variables listed above, the multivariate analyses demonstrated that a 10% increase in MPR was associated with a 4.7% decrease in total health care costs (p=0.032) and 2.4% decrease in disease-related health care costs to treat CML (p=0.357). For example, when MPR was improved from 75% to 85% for a patient, the annual total health care costs for the patient were reduced by $3100 and the annual CML related health care costs went down by $1200. *Conclusions.* Improved compliance with imatinib therapy is associated with decreased total health care costs and disease-related health care costs. Improving compliance to imatinib therapy may not only optimize clinical outcomes but may also reduce the overall societal burden of health care costs associated with cancer.

0166

MOLECULAR RESPONSE TO IMATINIB IN EARLY CHRONIC PHASE VERSUS LATE CHRONIC PHASE CML PATIENTS IN COMPLETE CYTOGENETIC RESPONSE: A COMPARISON AT 24 MONTHS OF 2 CLINICAL TRIALS OF THE GIMEMA-CML WP

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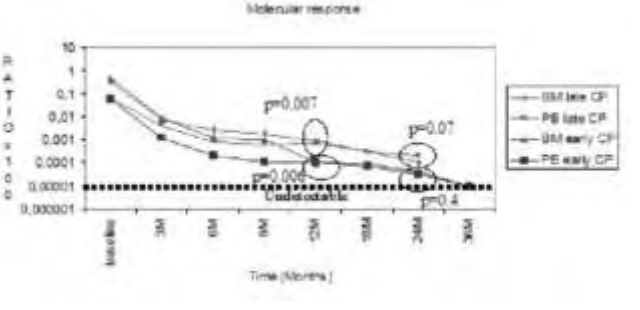
Background. The introduction of Imatinib (IM) has changed the current approach to the management of chronic myeloid leukaemia (CML). It is currently unclear whether patients (pts) treated with IM first-line treatment have a greater reduction of BCR-ABL transcript with respect to pts treated with IM after IFN-α failure, giving the same complete cytogenetic response (CCR). *Aims.*We sought to determine the differences in molecular response (MR) between early and late chronic phase (CP) pts with CML who achieved a CCR after treatment with IM at the standard dose of 400mg/d. We studied 2 different cohorts of pts in CCR: - 67/191 (35%) pts after α-Interferon (α-IFN) failure enrolled on the CML/002/STI571 protocol - 53/76 (70%) pts treated front line with a combination of IM and pegilated IFN-α (PEG-IFN) enrolled on the CML/011/STI571 protocol. *Methods.* Cytogenetic response was monitored on bone marrow (BM) metaphases and MR was assessed by real time RT-PCR (TaqMan) BM and peripheral blood (PB) samples, collected at baseline, 3, 6, 9 and 12 months during the first year, and every 6 months thereafter. MR was expressed as the ratio between BCR/ABL and β2-microglobulin (β2-M) x100. The lowest level of detectability of the method was 10-5. Negative results (i.e. undetectable transcript) were confirmed by nested PCR performed 4 times (sensitivity 10-6). For the purpose of this analysis, a major molecular response (MMR) was defined as a BCR-ABL/β2M value <0.0001%, which turned out to be roughly equivalent to a 3-log reduction and a complete molecular response (CMR) was defined as negative (undetectable) BCR/ABL levels confirmed by nested PCR.



Results. We observed a progressive decrease of the amount of BCR/ABL transcript in pts who achieved a CCR. At 24 months the median reduction in BCR/ABL transcript level was: a 3-log reduction in late CP pts; a 4-log reduction in early CP pts. In the latter group of pts MR was assessed also at 36 months. So we observed that 36 months after the first dose of IM and PEG-IFN pts who were still in CCR had the median value of BCR/ABL transcript of 0.00001% both in BM and PB. Therefore all these pts achieved a MMR. However only 8/53 (4%) pts were in CMR (undetectable BCR/ABL at least once as assessed by nested PCR). *Conclusions.* Although after 24 months of therapy front line treatment of CML pts with IM determines a major percentage of CCR in comparison of pts treated with IM after IFN failure (in our experience, 70% versus 35%, respectively) the differences in MR (reduction in BCR/ABL transcript level) observed in the 2 groups of pts were not significant. Nevertheless excellent results were obtained in both groups, with a median reduction in BCR/ABL transcript level of at least 3 log. In the pts treated with a combination of IM and PEG-INF a further reduction of BCR/ABL transcript (about another log) was observed at 36 months of treatment.

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HOMOHARRINGTONINE IS ASSOCIATED WITH A HIGH PROPORTION OF HEMATOLOGIC RESPONSE IN CML PATIENTS WITH HEMATOLOGIC FAILURE TO IMATINIB

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Background and *Aims.* Imatinib mesylate (IM) is now the gold standard for first line therapy in patients with chronic myelogenous leukaemia (CML). Homoharringtonine (HHT) is an alkaloid obtained by an original hemi-synthetic process from Cephalotaxus. HHT having shown activity in CML, we studied its role as a salvage single or combination therapy in patients presenting with primary or secondary hematologic resistance. *Methods.* We retrospectively analyzed data from all our patients in chronic phase (CP) or accelerated phase (AP) CML who received HHT as a salvage therapy in order to achieve a complete hematologic response, aiming so to determine its role in the current context of the disease management. *Results.* In total, 15 CML (CP, n=10 and AP, n=5) patients started HHT between 09-2000 and 10-2004 for lack or loss of hematologic response in the two institutions. Main previous therapy was interferon α (IFN, n=6, including 3 patients with IFN + Ara-C), or IM (n=9, including IM 400 mg per day for 8 patients, and IM 600 mg per day for 1 patient). Sex ratio was 10M/5F. Median age at baseline for HHT was 57 years (range: 38-70). Median time from diagnosis was 49 months (range: 18-121). HHT was administered at 1.25 to 2.5 mg/m_ daily dose, as a continuous 24h infusion or as 2 divided doses via the subcutaneous route. The course durations were maximum 6 days when combined to IM (n=3) or Ara-C (n=7), and up to 14 days when used as a single therapy (n=5). Of these 15 patients, 11 (73.3%) achieved a complete hematologic response (CHR) after 2 courses in median (range: 1-6), including 1 patient in accelerated phase and 5 patients with BCR-ABL kinase domain (KD) mutations (E255K; M244V; F317L + K247R; V244Q; Y253H). Responding patients received HHT as a single therapy (n=3), or combined with IM (n=3) or Ara-C (n=5). One additional patient who was in AP returned to chronic phase after 2 HHT + Ara-C courses. There was no hematologic response in the 3 remaining patients. Two of them received HHT as a single therapy and 1 received HHT + Ara-C. Among the patients detected with a BCR-ABL KD point mutation, all CP patients obtained CHR and the mutated patient in accelerated phase (Q252H) did not respond to HHT. Nine patients (60%) experienced grade 4 hematotogic toxicity, 6 of them presenting anemia requiring blood transfusion (9 occurrences). There was no significant extra-hematological toxicity reported. *Conclusion.* HHT as a single or a combination therapy may still be of interest for treating CML patients in the tyrosine kinase inhibitors era in case of resistant disease. Indeed, hematologial responses to HHT have been obtained in case of BCR-ABL KD mutations. The role of HHT alone or in combination will be refined with well-designed prospective studies.

0168

ACETYLOME AND PHOSPHOPROTEOME MODIFICATIONS OF IMATINIB SENSITIVE AND RESISTANT CML CELLS AFTER SHORT CHAIN FATTY ACID HISTONE DEACETYLASE INHIBITOR TREATMENT.

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Background. CML patients may become irresponsive to Imatinib because of resistance developed by amplification of the BCR-ABL genomic locus or by point mutations within the kinase domain of BCR-ABL. Innovative dual SRC/ABL kinase inhibitors with higher power against native and imatinib-resistant mutants of BCR-ABL give remarkable therapeutic benefits, but at least one mutation remains resistant to any kinase inhibitor (T315I). Given these evidences, the investigation of alternative therapeutic agents effective in CML still remains a subject of primary interest. *Aims.* We analysed whether HDACIs short chain fatty acids (SCFAs) valproic acid and butyrates were synergistic with imatinib. SCFAs acetylation of non-histone proteins is not well characterized, we thus compared the actylome and phosphoproteome of CML cells treated and not treated with HDACIs, alone and in combination with imatinib, by immunoproteomic techniques . *Methods.* The human CML cell lines K562, LAMA-84 S (Imatinib sensitive) and LAMA-84 R (Imatinib resistant), KBM-R and -S were grown in the presence of valproic acid at the escalating doses 0.2 mM to 2 mM or in the presence of butyric acid derivative D1 (0.2-1 mM) for 24 and 48 hrs. Apoptosis was monitored by annexin V test and propidium iodide uptake. Bcr-abl mRNA was measured by real time PCR. Bcr-Abl protein expression was determined by western blot with specific antibodies. Total cell proteins were separated by 2D electrophoresis (pH 3-11). We used a anti-pan-acetylated and anti-phosphotyrosine antibody for 2D WB, followed by matching with 2D gel and MALDI-TOF mass spectrometry for protein identification. *Results.* Apoptosis was induced time and dose dependently by VPA and D1. Imatinib was synergistic with both HDACIs in inducing apoptosis and cell proliferation arrest (WST-1-assay). VPA and D1 were able to induce a significant decrease in the number of copies of Bcr-abl mRNA both in sensitive and in resistant cells. A significant decrease in BCR-ABL protein expression was observed by WB of total cell lysates from CML cells. Twenty two proteins differentially acetylated were identified. At least two chaperone proteins were identified as target of acetylation after VPA and D1 treatment of CML cells, other targets were proteins involved in the synthesis and stability of RNA. Sixteen proteins differentially phosphorylated were identified. For 13 of these proteins the phosphorylation level was not significantly affected by HDACIs in resistant cells, while the combination of both Imatinib and HDACIs produced a considerable decrease of phosphorylation in both sensitive and resistant cell lines. This category includes: HSP90, HSP70, HOP1 and nucleophosmin. *Summary/Conclusion.* Short chain fatty acids are not the most powerful HDACIs, but have been used successfully in clinical trials. Our analysis show significant evidences of their effects on CML cells in terms of induction of apoptosis and arrest of CML cell proliferation. Further effects were observed on Bcr-Abl expression and modifications on both acetylome and phosphoproteome. This study characterizes proteome modifications provoked by SCFAs and may help to understand the molecular effects of different HDACIs in order to improve their use in combination with imatinib or new SRC/ABL inhibitors.

0169

IMATINIB THERAPY FOR CHRONIC MYELOID LEUKEMIA PATIENTS WHO RELAPSE AFTER ALLOGENEIC STEM CELL TRANSPLANTATION: A MOLECULAR ANALYSIS

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Background. Allogeneic stem cell transplantation (SCT) is to date the only curative therapy for CML patients, but relapse is still one of the major causes of failure. Discontinuation of the immunosuppressive therapy and donor lymphocyte infusion (DLI) is the treatment of first choice for CML recurrence after allografting, since induces durable remission in a substantial number of patients. Nonetheless, it requires the availability of the donor and may be associated with severe graft-versus-host-

disease (GvHD) and/or marrow aplasia. In the last years, there have been several reports of promising activity of imatinib in CML recurrence after allografting. *Aims.* In order to better define the role of imatinib in this setting, we report an extended molecular follow-up of 16 CML patients treated with imatinib while in relapse after allogeneic SCT. *Methods.* Patients underwent allogeneic, non T-cell depleted, standard conditioning regimen SCT. At evidence of relapse, 5 patients were in immunosuppressive therapy, which was discontinued. At start of imatinib, five patients were in hematological relapse, Nine had a cytogenetic relapse and two a molecular relapse. *Results.* Median follow-up is 33 months (range 12-45). All patients achieved a complete cytogenetic response (CCgR) within 12 months. Molecular response was evaluated in bone marrow and/or peripheral blood samples at start of imatinib therapy and every 3 months during treatment by a standardized real-time quantitative PCR (RTQ-PCR) method and/or by qualitative *nested* PCR. Complete molecular response (CMR) was defined as reduction of BCR-ABL/B2 Microglobulin below 0.00001 or negativity of qualitative *nested* PCR in bone marrow samples. Eight patients achieved and maintained a stable CMR. In seven patients, CMR has been achieved but lost at least once during follow-up. In these patients, median duration of longer CMR was 12 months (range: 3-24). All patients are in ongoing treatment with imatinib except for one patient who discontinued the therapy 8 months ago and maintain a CMR. No further treatment was administered in all but two patients, who received DLI after the achievement of CMR. Chimerism analysis has been performed by VNTR analysis or FISH in 8 patients. Prior to imatinib therapy, full donor chimerism was lost in 7 cases and was fully recovered after the achievement of CMR. *Conclusions.* In our experience, response rate to imatinib is comparable with that expected from treatment with DLI alone. All patients achieved cytogenetic and molecular responses, which were associated with reconstitution of full donor chimerism without increasing of GVHD. Moreover, no major side effects were observed. Although no direct comparison may be made, the data suggest that in our patients percentage of and time to molecular response to imatinib appeared to be better than in newly diagnosed CP CML patients, possibly due to residual GVL effect. An important observation is that even patients relapsed in advanced phase of disease obtained durable molecular responses (median duration of CMR: 20 months, range:6-24). Compared to other therapeutic approaches, our experience confirms that imatinib is effective and feasible, with a very high overall response and a manageable side-effects profile, at least in the short-term.

0170

INFLUENCE OF CYP3A4 ACTIVITY ON IMATINIB RESPONSE IN PATIENTS WITH CHRONIC MYELOID LEUKEMIA

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Background. Imatinib, also known as Gleevec/Glivec, is a potent Bcr-Abl tyrosine kinase inhibitor currently used for the treatment of chronic myeloid leukemia (CML) patients. Imatinib induces complete cytogenetic responses (CCR) in the majority of patients with CML in chronic phase (CP). However, a subgroup of patients is refractory at the cytogenetic level. Imatinib is mainly metabolised via CYP3A4 although several other CYP enzymes have been shown to be involved. The main metabolite CGP74588 a product of CYP3A4 is pharmacologically active, and shows similar potency and selectivity as imatinib. However, the influence of the metabolic pathway on the effect of imatinib and the activity of the metabolites need to be further investigated.

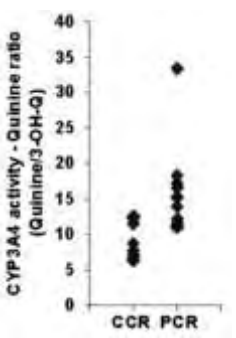

Figure 1. CYP3A4 activity and Imatinib response.

Aims. The aim of this study was to investigate the role of the drug metabolising enzyme i.e. the CYP3A4 activity *in vivo*, for the response to imatinib treatment. *Methods.* 16 chronic myeloid leukemia patients were included in the study. To assess the *in vivo* CYP3A4 activity the patients were given 250 mg of quinine, 16 h later a blood sample was collected and the ratio of quinine/3-hydroxyquinine was measured using HPLC. The response of imatinib treatment was evaluated by cytogenetic analysis and the patients were divided into CCR within nine months and partial cytogenetic responders (PCR) who had failed to achieve a CCR. *Results.* Patients with CCR showed significantly (Mann-Whitney U-test, p=0.013) higher CYP3A4 activity (low Quinine ratio, mean = 9.4, SD = 2.7) compared to patients that were PCR (high Quinine ratio, mean = 16.6, SD = 6.8), see figure. *Conclusions.* CML patients with high CYP3A4 activity respond better to imatinib treatment than patients with low activity. Clinically, it would be advantageous to identify such patients a priori, since they may benefit from more aggressive therapy. This also indicates that the effect and potency of the metabolites might be of clinical importance.

0171

IMATINIB 400 MG IN LOW SOKAL RISK CML PATIENTS: EARLY RESULTS OF AN OBSERVATIONAL, MULTICENTRIC TRIAL OF THE GIMEMA CML WP

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Background. Imatinib 400 mg is the established first line treatment of chronic myeloid leukemia (CML) in chronic phase. The efficacy of imatinib in early chronic phase has been demonstrated by multicentric randomized controlled trials like the IRIS trial (O' Brien et al NEJM 348:11, 2004). Large multicentric studies aimed to evaluate the impact of imatinib 400 mg outside strictly monitored trials are not yet available. *Aims*. The GIMEMA (Gruppo Italiano Malattie Ematologiche dell'Adulto) CML Working Party opened in January, 2004, an observational study (serial n. CML/023) to investigate the efficacy of imatinib 400 mg in newly diagnosed CML patients. *Methods.* Clinical and anagraphical data were collected through a web-based system. Responses were evaluated at fixed time-points during treatment. Hematologic: continuously; cytogenetic at 6 and 12 months (local labs); molecular response at 3, 6 and 12 months.Peripheral blood samples for quantitative molecular analysis (RT-Q-PCR, Bcr-Abl/Abl × 100 - Taqman) were centralized in Bologna at 3, 6 and 12 months. *Patients.* Overall, 54 italian centers enrolled 209 (188 evaluable) low Sokal risk patients between January 1, 2004 and November, 2005. Median age was 44 yrs (range 20-69), 117 male and 71 females. 188 patients are evaluable for response at 3 months, 151 at 6 months and 84 at 12 months. The median observation time is 6 months. *Results.* At 3 months, 95% of the patients reached a stable complete hematologic response. At 6 months, 81% of the evaluable cases obtained a complete cytogenetic response (100% Ph-neg, CCgR). A major molecular response (MMR) defined as a Bcr-Abl/Abl × 100 ratio < 0.1%, was shown in 51% of CCgR patients. At 12 months, the CCgR rate was 88% and the MMR rate in CCgR patients was 57%. At 12 months, 4% of CCgR cases showed a undetectable level of transcript (ratio Bcr-Abl/Abl × 100 < 0,00001) . With this short observation period, only 1 pt progressed to accelerated/blastic phase, while 2 patients were censored at the time of allogenic stem cells transplantation. SUMMARY AND *Conclusions.* The preliminary evidences of our observational trial confirm that imatinib 400 mg is a highly effective treatment for CML in early chronic phase, as far the CCgR and MMR response rates. 201 low Sokal risk patients were enrolled in the IRIS trial and received imatinib as first line treatment. The CCgR rate within 12 months was 76% with 66% of patients reaching a MMR (defined as reduction of Bcr-Abl transcript level > 3 logs; control gene Bcr) (T Hughes *et al.*, NEJM 349:15, 2003). Our results (81% and 88% CCgR rate at 6 and 12 months, 51% and 57% MMR at 6 and 12 months) compare favourably with the IRIS trial results.

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0172

IMATINB AND AGING: PRELIMINARY RESULTS OF A SUB-ANALYSIS WITHIN 3 TRIALS OF THE GIMEMA CML WP

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Background. Older age constitutes a poor prognostic variable in Ph+ CML patients, across treatment modalities . Age is included and acts as negative factor in the staging systems most employed (Sokal and Euro). Older patients have been generally excluded from most of the trials employing interferon, due to its toxyc effects. Few studies investigated the effect of imatinib in older patients:Cortes et al (Cancer 98, 2003), based on a single center casistic (187 early CP patients overall, 49/187 older than 60 yrs), treated at doses between 400 and 800 mg, showed no difference in response and outcome, observation which needs to be confirmed. *Aims*. To investigate the effects of age on response and compliance to imatinib. *Methods.* A sub-analysis within 3 simultaneously running trials of the GIMEMA (Gruppo Italiano Malattie Ematologiche dell'Adulto) CML WP (n.CML/021, phase II - ima 800 in intermediate Sokal risk; CML/022, phase III- ima 400 vs 800 mg in high Sokal risk, n. CML/023, observational - ima 400 mg) have been performed. Overall, 404 patients have been enrolled (January, 2004- November, 2005): at enrollment 85/404 (21%) were > 65 yrs (median age 71, range 65-85) and 319/404 (79%) < 65 yrs (median age 46, range 18-64). Sokal risk distribution was different between the 2 groups: low Sokal risk cases were 15% in older cohort vs 54% in younger cohort (p<0,01). 21% of older and 22% of younger pts received high dose (800 mg) of imatinib front-line. Timing of response evaluation: hematologic, continuously; cytogenetic, at 6 and 12 months; molecular, at 3, 6 and 12 months.PB samples for quantitative analysis (RT-Q-PCR, Bcr-Abl/Abl × 100 - Taqman) were centralized in Bologna at 3, 6 and 12 months. *Results.* The numbers (%) of evaluable cases (older/younger) at 3,6 and 12 months were: 85/319 (100%/100%), 59/251 (69%/79%) and 27/141 (32%/44%). At 3 months, both groups achieved a 93% complete hematologica response (CHR) rate. At 6 months, the complete cytogenetic response (CCgR) rates were (older/younger) 66%/80% (p=0,39). The major molecular response (MMR, defined as a Bcr-Abl/Abl × 100 ratio < 0.1%) rates (CCgR only) were 67%/49% (p= 0,06). At 12 months, CCgR rates were 81%/88% (p= 0,67) and MMR 50%/60% (p= 0,04). With a median observation time of 6 months, 1 pt (1%) of older cohort and 4 (1%) of younger cohort progressed to accelerated/blastic phase. *Summary and Conclusions.* This subanalysis was generated from 3 trials with different aims and dosages of imatinib. The observation period is still short. However, it is noteworthy that, notwithstanding a worsen risk distribution of older cases (15% low risk vs 54% for younger), results at 6 and 12 months are comparable. The only significative difference was demonstrated for MMR at 12 months. Consequently, we may foresee that the long-term survival and progression free survival will not differ between the 2 groups. *Acknowledgments. Supported by: COFIN 2003, FIRB 2001, A.I.R.C, C.N.R., Fondazione del Monte di Bologna e Ravenna, LeukemiaNet, A.I.L.*

0173

BCR-ABL REDUCES CCN3 EXPRESSION THEREBY EVADING NEGATIVE GROWTH REGULATION

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Background. Chronic Myeloid Leukemia (CML) is characterized by expression of the constitutively active BCR-ABL tyrosine kinase. Previously, we have identified downregulation of the negative growth regulator, CCN3, as a result of BCR-ABL kinase activity and detected reduced CCN3 expression in human CML cell lines and primary human CML cells. *Aims.* To identify the relationship between BCR-ABL and CCN3 expression and the functional consequence of expressing CCN3 in BCR-ABL+ cells. *Methods.* Real-time PCR was used to examine the relationship between BCR-ABL and CCN3 expression in human K562 cells using siRNA directed against BCR-ABL or the BCR-ABL tyrosine kinase inhibitor, Imatinib. CCN3 function was investigated in K562 cells transfected with vector or vector containing CCN3 construct by assessing cell growth using flow cytometry and colony formation on methyl cellulose. *Results.* Parental K562 cells showed high expression of BCR-ABL whilst CCN3 expression was present at low levels. Treatment with siRNA directed against BCR-ABL resulted in a 3.7 fold decrease in BCR-ABL and 6.1 fold increase in CCN3 expression (mean Ct change 1.9±0.2 and 2.6±0.5 for BCR-ABL and CCN3 respectively, n=3, p=0.001). Similarly, K562 cells treated with imatinib (1 micromolar) for 96 hours showed a 5.9 fold decrease in BCR-ABL expression and a 4.2 fold increase in CCN3 expression (mean Ct change 2.5±0.1 and 2.1±0.2 for BCR-ABL and CCN3 respectively, n=3, p=0.001). To investigate CCN3 function, we expressed CCN3 in BCR-ABL expressing cells. K562 cells were transfected with either the pCb6+ vector or pCb6+ vector containing the CCN3 construct. Cell cycle analysis was performed: CCN3 expression in BCR-ABL+ cells resulted in an accumulation of cells in the subG0 phase of cell cycle, indicative of cell death (mean for subG0 9.9%±4.6 and 21.8%±0.7 for the pCb6+ vector alone and pCb6+ vector containing CCN3 construct respectively). In addition, CCN3 expression reduced the clonogenic capacity of BCR-ABL+ cells. K562 cells transfected with the pCb6+ vector containing CCN3 construct formed significantly fewer colonies on methyl cellulose in comparison to cells that had been transfected with the pCb6+ vector alone (n=3, p=0.027). *Conclusions.* This study demonstrates a reciprocal relationship between CCN3 and BCR-ABL expression. CCN3 is known to be a negative growth regulator and increased expression of CCN3 in BCR-ABL+ cells inhibits proliferation and decreases clonogenic potential. Thus CCN3 down-regulation mediated by BCR-ABL offers growth advantage to hematopoietic cells.

0174

THE PERSISTENCE OF P190 BCR-ABL TRANSCRIPTS IS ASSOCIATED WITH LOWER PROBABILITY OF MOLECULAR RESPONSE TO IMATINIB IN EARLY AND LATE CHRONIC PHASE CML PATIENTS.

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Background. It has been demonstrated that the about 70% of p210CML patients in chronic phase (CP) at diagnosis co-expressed p190 BCR/ABL transcripts, although at a much lower level. In this study we have assessed by real-time quantitative reverse transcription PCR (qRT-PCR) for the co-expression of p190 and p210 BCR/ABL transcripts 1) at diagnosis in previously untreated CP-CML patients, and 2) during the treatment with imatinib in 2 different groups, those with previously untreated disease (early CP-CML) and those who previously failed IFN therapy (late CP-CML). The clinical relevance of p190 BCR-ABL monitoring in CML pts under Imatinib is still unknown. *Materials and Methods.* Bone marrow (BM) samples were obtained from 83 pts with CP-CML treated with Imatinib at a conventional oral dose. These included 192 samples from 43 pts with late CP-CML and 140 samples from 40 pts with early CP-CML. Median follow-up was 18 (3-58) and 39 (12-58) months for early and late CP-CML, respectively. As part of a diagnostic work-up, BM samples were assessed for expression of both p210 and p190 BCR/ABL levels by qRT-PCR using a TAQ-Man system (ABI Prism 7700) for BCR-ABL and ABL genes. The median number of BM assessment was 3 (2-6) for early CP-CML and 4 (2-10) for late CP-CML. A major molecular response (MMR) was defined as p210 BCR-ABL/ABL ratios less than 0.05%. *Results.* Co-expression of p210 and p190 BCR-ABL transcripts at diagnosis was found in 22/25 (88%) of CP-CML at diagnosis. There was a significant correlation between the number of p190 and p210 transcripts only in samples of the early CP-CML (Canonical Correlation R=0.688, p<0.00001), but not of the late CP-CML (R=0.0234 p=NS) and of all 332 samples (R=0.021 p=NS), suggesting a residual expression of p190 transcripts in some BM samples with low p210 transcripts. In fact, we found that 13/108 (12%) BM samples with p210 BCR-ABL/ABL ratio<0.05% showed a co-expression of p190 BCR-ABL transcripts [median value=0.014% (0.0014%-0.063%)]. A MMR was obtained in 20 pts (50%) and 20 pts (46%) with early and late CP-CML, respectively. To test if the persistence of p190 transcripts during the follow-up was predictive of MMR, we divided CML pts in two groups, those with 0 or 1 p190 BCR-ABL+ samples (group 0-1) and those with 2 or more positive samples (group≥2). We found that for late CP-CML pts the group ≥2 showed a significant lower probability to obtain MMR compared to group 0-1 [17/24 (71%) vs 5/19 (26%) p=0.0039)]. The same result was observed for early CP-CML [15/21 (71%) vs 6/18 (33%) p=0.017)]. *Conclusions.* In this study, we confirm the frequent co-

expression of p190 and p210 transcripts by CP-CML patients at diagnosis. However, some patients with low levels of p210 transcripts continue to display p190 expression. The persistence of p190 signals despite the 2-3log fall in p210 BCR-ABL levels may be of prognostic value. The significance of the lack of correlation between p190 and p210 transcript levels warrants further investigations and may disclose unfolded biological relevance.

0175

IMPACT OF BCR/ABL GENE EXPRESSION ON THE PROLIFERATIVE RATE OF DIFFERENT SUBPOPULATIONS OF HEMATOPOIETIC CELLS IN CHRONIC MYELOID LEUKEMIA

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Background and Aims. Despite the effects of BCR/ABL on cell proliferation, no study has been reported so far in which the proliferative rate of different hematopoietic cell compartments from chronic myeloid leukaemia (CML) has been compared to normal bone marrow (NBM). In order to gain further insight into the potential impact of BCR/ABL gene expression on leukemic CML cells, we compared the proliferative rate of different BM cell subpopulations from CML patients and normal subjects and explored the correlation between the proliferation of each cell population from CML with BCR/ABL gene expression in highly-purified fractions of BM cells. *Methods.* A total of 26 BM samples corresponding to 15 patients diagnosed of CML and 11 NBM were studied. The proportion of S+G2/M cells was analyzed on CD45/CD19/DRAQ5, CD34/CD117/DRAQ5, CD11b/CD13/DRAQ5, CD36/CD14/DRAQ5 and HLA-DR/CD123/DRAQ5 stained BM cells. Fluorescence activated cell sorting (FACS) of BM cell populations from CML patients (n=7) was performed using a FACSAria flow cytometer (BDB) and the FACSDiVa software (BDB). A CD38/CD34/CD19/CD13/CD11b/CD45 6-colour staining was used to sort $CD38^+/CD34^+/CD19^-$ myeloid hematopoietic stem and precursor cells, CD34- myeloblasts (SSChigh/CD45low/CD34-/$CD11b^-/CD13^+$ non-autofluorescent cells), promyelocytes (SSC^{high}/$CD45^{low}$/CD11b+/CD13+ non-autofluorescent events), myelocytes/metamyelocytes ($SSC^{high}/CD45^{low}/CD11b^{high}/CD13+^{low}$ non-autofluorescent events) and bands/neutrophils (SSC^{high}/ $CD45^{low}$/ CD11bhigh/CD13high non-autofluorescent cells). Quantitative-real time PCR (RQ-PCR) analyses were performed on whole BM samples and purified cell populations in MicroAmp 96-well optical plates on an ABI PRISM 7700 sequence detection system (PE Applied BioSystems, Foster City, CA) and the results expressed as normalized BCR/ABL copy numbers (NCN) per one copy of the GUS gene $\times 10^4$. *Results.* Overall, our results showed similar proliferative indices in CML patients and NBM. However, $CD34^+$ myeloid precursors from CML patients displayed an increased proportion of S+G2/M-phase cells (p=0.04), while no significant differences were found between CML and NBM for other BM cell subsets analyzed. In FACS-sorted BM cells, decreasing levels of BCR/ABL mRNA were found from $CD34^+/CD38^+$ myeloid precursors to myeloblasts; BCR/ABL expression increased afterwards with a peak at the myelocyte/metamyelocyte stage, decreasing in the more mature band/neutrophil compartment. Unexpectedly, BCR/ABL gene expression showed an inverse correlation with the proportion of S+G2/M-phase cells (R=-0.33; p=0.04). *Conclusions.* These results suggest that in CML, BCR/ABL expression is associated with an increased proliferation of CD34+ myeloid HPC but not of other more mature myeloid precursors as also confirmed by the lack of direct correlation between the amount of BCR/ABL transcripts and the proportion of S+G2/M-phase cells.

0176

SURVIVIN AND CIAP-1 GENE EXPRESSION IS LINKED TO CHRONIC MYELOID LEUKEMIA PROGRESSION AND POOR RESPONSE TO IMATINIB

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Background. Chronic myeloid leukemia (CML) is a myeloproliferative disease in which bcr-abl oncogene enhances survival of leukemic cells through modulation of proapoptotic and antiapoptotic molecules. The IAPs (inhibitor of apoptosis proteins) are a family of caspase inhibitors that block the execution phase of apoptosis. Overexpression of IAPs confers chemoresistance and, in some groups of cancer patients, is associated with a poor prognosis. Fully understanding the basic apoptotic pathway and its regulation in Bcr-Abl-positive cells will unveil more targets for manipulation, which can be translated into novel therapies. *Aim.*The objective of this work was to determine the IAPs gene expression (ciap-1, ciap-2 and survivin) in 15 healthy individuals and on 71 CML patients (20 chronic phase, 15 accelerated phase, nine blastic phase, 20 in cytogenetic remission and seven refractory patients post-imatinib). *Methods.* The iaps gene expression was performed in CML patient's peripheral blood mononuclear cells by quantitative real-time RT-PCR. *Results.* The results are expressed by relative expression e.g. ratio of investigated gene to the reference GAPDH gene. survivin and ciap-1 gene overexpression was observed in 61 (86%, p<0.001) and 55 (77%, p<0.001) patients, respectively. The survivin levels (mean/SD) were: 0.11/0.02 in controls; 0.53/0.14 in chronic (CP), 2.1/0.55 in accelerated (AP), 9.6/3.3 in blastic (BP) phases, 0.04/0.02 in CML remission and 18.08/8.0 Gleevec-refractory patients. The ciap-1 expression was 17.6/3.53 in controls; 31.02/5.9 in CP, 15.75/2.6 in AP, 57.23/15.49 in BP, 32/8.5 in CML remission and 59.04/12.05 in Gleevec-refractory patients. *Conclusions.* Therefore, there was an association between survivin (p<0.001) and ciap-1 (p<0.001) mRNA level with CML stage and response to imatinib. The ciap-2 gene expression observed in healthy controls and CML patients are similar (p>0.05). Taken together our results suggest that each IAP homologue has a different mechanism of action and because more than one member of this family may be overexpressed in CML, successful treatment strategies for this disease will be defined by the ability to block all of the IAP expressed or to associate its inhibitor with other therapies.

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0177

ABL KINASE DOMAIN MUTATIONS ARE IMPORTANT MECHANISMS OF RESISTANCE IN ASIAN PATIENTS WITH IMATINIB-RESISTANT CHRONIC MYELOID LEUKAEMIA

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Introduction. While the efficacy of imatinib in chronic myeloid leukaemia (CML) is without doubt, resistance remains a problem, especially in the advance phases. The most common mechanism of resistance is point mutations leading to amino acid substitutions in the Abl kinase domain and has been described mainly in the Western population. Little information is currently available if this mechanism is also common among Asian patients. *Methods.* Samples from 32 patients with suboptimal responses or progression on imatinib have been analysed so far. RNA was extracted from peripheral blood and complementary DNA synthesised using random hexamers. The Abl kinase domain was amplified using a two-step hemi-nested reverse transcriptase/polymerase chain reaction (RT/PCR) and the PCR products subjected to direct sequencing. *Results.* The median age was 47 (19-77) years with a equal sex distribution. There were 15 Chinese, 9 Indian and 8 Malay patients. Thirteen patients were in the chronic phase (CP), 14 in accelerated phase (AP) and 5 in blast phase (BP). The median duration of imatinib treatment was 27 (7-65) months. A total of 20 mutations were detected in 14 patients, with 8 located in the ATP-binding loop, 4 in the imatinib-binding site, 2 in the catalytic domain and 2 in the activation loop. The remaining 4 were in other sites within the Abl kinase, of which one is a novel mutation (T240A). This new mutation will be verified by amplifying the ABL alleles to exclude polymorphisms. Four patients had more than one mutation. Of these 14 patients, 12 were enrolled into clinical trials with second generation Abl

kinase inhibitors (dasatinib, Bristol-Myers Squibb or AMN107, Novartis). Mutation testing was also performed in 6 patients after 3 months of dasatinib (see Table).

Table 1. Amount of shading = rel size of mutant clon.

Patient	Phase	Mutation pre-dasatinib	3 months post-dasatinib	Response to dasatinib at 3 months
1	AP	F359V ●	F359V ○	Haem progression
		T315I ◑	T315I ●	No cytogenetic response
2	AP	T240A ●	T240A ◑	Complete haem response
		G250E ○	G250E ◑	No cytogenetic response
		Y253F ●	Y253F ◑	
		H396R ◑	H396R ○	
3	AP	H396R ●	H396R ●	Complete haem response
				No cytogenetic response
4	AP	E255K ●	E255K ●	Complete haem response
				Minor cytogenetic response
5	CP	E355G ●	E355G ◑	Complete haem response
				Minor cytogenetic response
6	CP	E255V ●	E255V ○	Haem progression
		T315I ○	T315I ●	No cytogenetic response

Haematologic progression was observed with the development of the T315I mutant in 2 patients and in 1 patient, a minor cytogenetic response was associated with a relative reduction in size of the mutant clone. *Conclusions.* Our study shows that Abl kinase mutations are common in Asian CML patients resistant to imatinib. Currently, mutation testing is only available in a few laboratories across the continent. It is therefore important that screening for mutations be performed routinely in imatinib-resistant Asian CML patients as this will have an impact on therapeutic decision making.

0178

INCREASED ANGIOGENESIS IN PATIENTS WITH CHRONIC MYELOID LEUKEMIA: CLINICAL AND MORPHOLOGICAL ANALYSIS

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The degree of intratumoral neo-vascularization as well as the expression of some proangiogenic factors is of prognostic significance in some solid tumors. It is still to be elucidated the possible prognostic role of increased angiogenesis in patients with haematological diseases. The aims of the present study are: (1) to analyze the neo-vascularization of bone marrow in patients with chronic myeloid leukemia (CML); (2) an assessment of bone marrow cellular expression of Vascular endothelial growth factor (VEGF) and its receptor VEGFR-2 (KDR) as well as the plasma levels of VEGF in CML; (3) to analyze the correlation dependency of angiogenic factors with the prognostic and biologic markers of the disease. A totally of 37 patients with CML as well as 30 healthy individuals were analyzed for VEGF plasma levels by using ELISA technique. Immunohistochemical methods were applied to visualize the vascular structures as well as the VEGF/KDR cellular expression in 17 trephine biopsies from newly diagnosed patients with CML and in 15 normal bone marrows. We observed that the mean vessel count per field was 3.13 per 0.0625 mm^2 in normal bone marrows vs. 24.6 per 0.0625 mm^2 in CML ($p<0.001$).

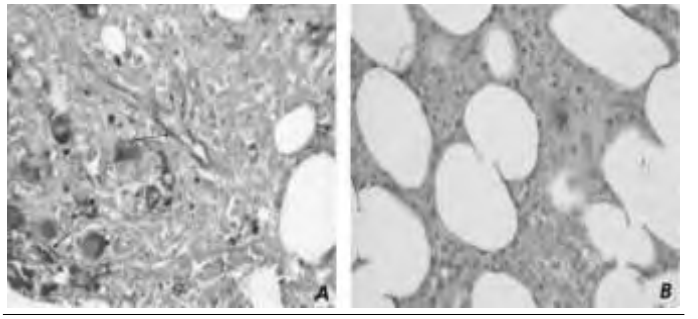

Figure 1. Increased MVD in CML bone marow (A) comparing to the normal (B) case. (Von WF immunostaining)

The VEGF/KDR cellular expression levels were nearly 5-fold that in normal control samples and the VEGF plasma levels were significantly higher in CML group (Mean 429 vs. 36.8 pg/ml; $p<0.001$). A good correlation was found between plasma VEGF and platelets as well as leucocytes but not with the blast per cent and Hasford prognostic score. Likewise, plasma VEGF levels could not predict the acceleration of the disease, moreover, their levels decline with the progression of CML. We found a good correlation between MVD and cellular VEGF/KDR expression but only cellular KDR is in a significant correlation with Hasford prognostic score. According to our results the high MVD, VEGF and KDR expressions indicate that angiogenesis is an inevitable event in the pathophysiology of CML. The complex angiogenic assessment of bone marrow provides more reliable information about the occurrence and the significance of this process in CML than using VEGF plasma concentration alone. That is to say, the precisely defined patients with CML, which have a high rate of angiogenic activity in bone marrow, could benefit by angio-suppressive therapy.

Non-Hodgkin's Lymphoma - Clinical I

0179

A PHASE II, MULTICENTER, SINGLE-AGENT STUDY OF BENDAMUSTINE HCL IN PATIENTS WITH RITUXIMAB-REFRACTORY INDOLENT B-CELL NON-HODGKIN'S LYMPHOMA

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Background. Bendamustine HCl (TREANDA™) is a novel, multifunctional, hybrid, cytotoxic agent with novel mechanisms of action. Unlike other commonly used chemotherapeutic agents, bendamustine induces durable DNA damage resulting in rapid cell death in apoptosis-resistant cancer cell lines through the apoptosis-independent pathway of mitotic catastrophe. Studies have reported single-agent activity in patients with relapsed/refractory non-Hodgkin's lymphoma (NHL), chronic lymphocytic leukemia, multiple myeloma, and breast cancer. *Aim.* This study evaluated the efficacy and safety of bendamustine in patients with NHL who had relapsed and were refractory to prior rituximab treatment. *Methods.* This Phase II, multicenter study enrolled patients with relapsed, indolent, or transformed, rituximab-refractory B-cell NHL. Rituximab-refractory disease was defined as no response or progression within 6 months of completing rituximab treatment. Patients received bendamustine 120 mg/m^2 IV over 30 to 60 minutes on days 1 and 2 every 21 days for 6 cycles. Response was measured using the International Working Group criteria. *Results.* The intent-to-treat (ITT) population consisted of 77 heavily pretreated patients (53% male) with a median of 4 prior systemic therapies (range: 1-9), enrolled in 14 sites in the US and Canada. Median age of patients was 63 years (range: 38-84), and 87% had stage III/IV disease. Indolent histologic phenotype was seen in 82% of patients, while 18% had transformed disease. The overall objective response rate (ORR) in the ITT population was 74%; 38% had a complete response (CR) or unconfirmed complete response (CRu), 36% had a partial response (PR), 6% had stable disease (SD), and 16% had progressive disease (PD) (4% unknown). In the 14 patients with transformed disease, the ORR was 64%, with 14% CR + CRu, 50% PR, 7% SD, and 29% PD. By comparison, in the 62 patients with indolent lymphoma, the ORR was 78%, with 44% CR + CRu, 34% PR, 6% SD, and 13% PD (3% unknown). The median duration of response was 7.7 months for all patients, 2.5 months for transformed patients, and 8.4 months for indolent patients. The most common nonhematologic adverse events were nausea (72%), fatigue (47%), and vomiting (41%). Most of these events were grade 1 or 2; mild alopecia rarely observed (5%). The primary hematologic toxicity was reversible myelosuppression, with grade 3 or 4 adverse events including neutropenia (53%), thrombocytopenia (25%), and anemia (12%). MDS reported with similar frequency to the published incidence in this population. *Conclusions.* Single-agent bendamustine HCl (TREANDA™) was well tolerated and produced a high rate of durable objective responses, despite unfavorable prognostic features, in heavily pretreated rituximab-refractory, indolent and transformed NHL patients. The findings suggest that bendamustine may be an effective treatment for this patient population, which currently has very few treatment options. A Phase III trial with bendamustine as a single agent in patients with rituximab-refractory indolent NHL is ongoing.

0180

CLINICAL FEATURES AND PROGNOSTIC ASSESSMENT OF NODAL MARGINAL ZONE B-CELL LYMPHOMA, A RARE DISEASE WITH FOLLICULAR-LIKE BEHAVIOUR

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Background. Primary nodal marginal zone B-cell lymphoma (MZL) is a rare entity recognized by the WHO classification. Diagnosis requires a lymph node localization in the absence of prior or concurrent involvement of extranodal sites. Most studies reported so far focus mainly on histopathology, while the clinical features and outcome of this uncommon lymphoma remains less defined. *Aim.* To define the clinical features and to assess prognosis of primary nodal marginal zone B-cell lymphoma. *Methods.* We studied a series of 47 newly diagnosed patients with primary nodal marginal zone B-cell lymphoma. Diagnosis was made on histologic examination of lesional tissues integrated with immunohistochemical data. No patient showed MALT or splenic localisation of lymphoma at diagnosis. *Results.* Patients: 17 males and 30 females, median age 63 years (25-79) with 64% aged more than 60 years. 13% of patients had stage I disease, 10% stage II, 32% stage III, 45% stage IV (bone marrow involvement). 11% had peripheral blood involvement, 11% had bulky disease, 15% B symptoms, 6% ECOG score^3 2. 23% had hemoglobin <12 g/dL. LDH was above normal in 15% and b2-microglobulin in 45%. 11% had an autoimmune *Background.* HCV serology was positive in 24% (9/38). With the IPI score 37% ranked in the low risk, 22% in the low-intermediate, 35% in the intermediate-high, and 7% in the high risk category. Using the FLIPI score, 33% were classified as low risk, 34% as intermediate risk, and 33% as high risk. After treatment, 57% achieved a complete response and 24% a partial response, for an overall response rate of 81%. At a median follow-up of 2.6 years, no patient developed splenic or MALT involvement. 5-years and 10-years OS is 69% (95% CI 52-86%).

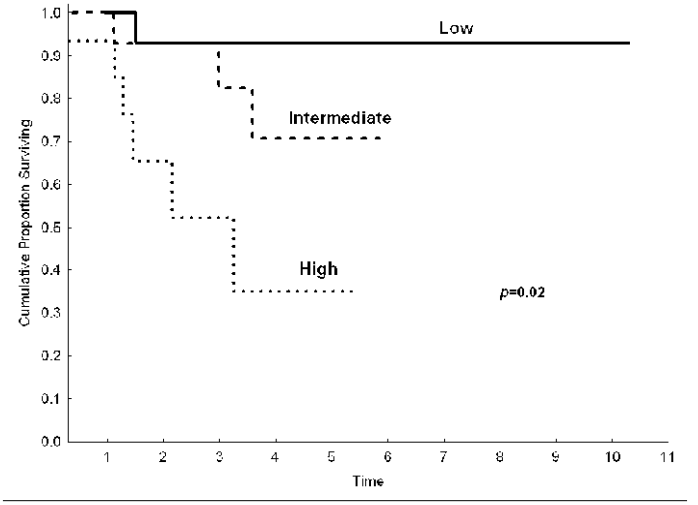

Figure 1. OS of primary nodal MZL according to FLIPI.

Death occurred in 10 pts (related to NHL in 9, to another neoplasm in one). In univariate analysis the following factors were associated with shorter event-free survival (EFS): B symptoms (p=0.001), high vs intermediate vs low risk FLIPI score (p=0.009). The following factors were associated with worse overall survival: high vs intermediate vs low risk FLIPI score (p=0.02) (Figure 1), age > 60 years (p=0.05), LDH above normal (p=0.05). HCV positivity was of borderline significance (p=0.06).In multivariate analysis hemoglobin < 12 g/dL (p=0.02, HR 14.3) was predictive of shorter EFS. Concerning overall survival, only the FLIPI retained statistical significance in predicting a worse outcome (p=0.02, HR 3.5). Positive HCV serology was of borderline significance (p=0.06, HR 4.4). *Conclusions.* among marginal zone neoplasms, primary nodal marginal zone lymphoma appears a distinct disorder with an indolent behaviour. The association with HCV infection (25%) is particularly high in comparison with non-marginal zone lymphomas. Considering the prognostic assessment of this rare disease, the FLIPI score is effective in detecting patients at worse prognosis with the same power as in

follicular lymphoma. Thus, the application of the FLIPI may be of clinical value for treatment decision also in primary nodal marginal zone lymphoma.

0181

PRELIMINARY EVALUATION OF EFFICACY AND TOXICITY OF TWO DOSES SCHEDULES OF BORTEZOMIB PLUS R-CHOP REGIMEN IN FRONT-LINE B LYMPHOMA PATIENTS

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Background. Bortezomib (Velcade®) is the first of its class of proteasome inhibitors tested in humans that showed promising activity as single agent in several tumor types, and especially in hematologic malignancies, in phase II studies. Only few reports have been made with combination chemotherapy. *Aims.* In March 2004, we initiated a phase II study with the bortezomib and R-CHOP regimen to evaluate efficacy and toxicity of the combination in NHL B patients. *Methods.* All patients received 6 cycles of standard Rituximab-CHOP (day 21= day1). Patients were randomized between two doses schedules of administration of bortezomib: A (bi-weekly: day 1,4,8 and 11), B (weekly: day 1 and 8). For the first 24 patients (step 1), Velcade® was administred at 1mg/m^2 in group A and 1.3 mg/m^2 in group B. For the next 24 patients (step 2), in absence of severe toxicity in step 1, it was planned to increase velcade at 1.3 mg/m^2 in group A and 1.6 mg/m^2 in group B. *Results.* We report here the safety results on 29 patients, there were 11 females, 18 males, with a median age of 59 years old (32-76). Histology: 2 Lymphoplasmocytic Lymphoma; 5 Marginal Zone Lymphoma; 8 Follicular Lymphoma; 3 Follicular Lymphoma with histological Transformation; 3 Mantle Cell Lymphoma and 8 Diffuse Large B Cell Lymphoma without adverse factor (IPI=0). Performance status > 2 : 0 ; LDH > N : 9 ; number of extra-nodal sites > 1 : 11. 27 patients received 6 cycles (1 patient was in progression after 5 cycles and 1 patient did not receive the 6th cycle). In step 1, group A, 90 to 100% of scheduled dose of bortezomib was administered. For group B it was 99 to100%. In step 2, group A, 78 to 100% of scheduled dose of bortezomib was administered. It was 100% for group B. Dose reduction were made after cycle 4. G-CSF and EPO support was used when necessary. Grade 3-4 hematotoxicity (per cycle) occurred in 13% for platelets, 43% for leukocytes. There was no red blood cells transfusion. The neurological grade 3-4 toxicity occurred in 5 patients. All occurred in the biweekly group (step 1 n=2/12; step 2 n=3/3) and none in the weekly group. The others observed major toxicities grade 3-4 were nausea 1/29, diarrhoea 1/29, 1 serious infections and 1 angina pectoris. After 6 cycles, the overall response rate was 97% (28/29) with 26 patients in CR/CRU (90%

Group	*Step*	*Stable RC*	*Stable RCU*	*Partial Remission RP*	*Progression*
Bi-weekly (n=15)	1 (n=12) 2 (n=3)	5	9	1	0
Weekly (n=14)	1 (n=12) 2 (n=2)	8	4	1	1

0182

CLINICAL FEATURES AND TREATMENT OUTCOMES OF ANGIOIMMUNOBLASTIC T-CELL LYMPHOMA

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Angioimmunoblastic T-cell lymphoma (AITL) is a rare subtype of lymphoma, making up only 1% to 2% of non-Hodgkin's lymphomas. The objective of this study was to investigate clinical features and treatment outcomes in patients with AITL. From September 1990 to June 2005, 65 patients diagnosed as AITL were included in the analysis. About one half of patients presented with poor performance status (ECOG > or =2); 72.3% of patients were categorized as high intermediate or high-risk group according to IPI; and most of patients (95.4%) were diagnosed at advanced stage. At diagnosis, 27 patients (41.5%) presented with malignant pleural effusion; 22 patients (33.8%) had a skin involvement. The median overall survival for all patients with AITL was 15.1 months (95% CI, 6.7-23.5). The response rate of 50 patients who had been treated with primary anthracycline-based chemotherapy was 80.0%; 62.0% CR rate, 18.0% PR rate. After a median follow-up of 39.0 months (range 2.4-177.8) in 33 patients who had achieved a CR, 15 patients (45.5%) developed disease recurrence with median CR duration of 45.6 months (95% CI, 25.5-65.7). The median progression free survival of all patients was 7.1 months (95% CI, 2.8-11.4). High dose chemotherapy followed by autologous stem cell transplantations were conducted in 4 patients for salvage therapy, which results showed 3 CR, 1 treatment-related mortality. The adverse prognostic factor for survival was only high IPI score in multivariate analysis. In conclusion, although AITLs showed a better response to the conventional anthracycline-based chemotherapy than the others, its response durations were short, therefore the chemotherapy regimen for AITL should be modified or intensified like a high dose chemotherapy followed by autologous stem cell transplantation especially in responsive patients.

0183

AUTOIMMUNE DISEASES IN PATIENTS WITH MALT-LYMPHOMA: CHARACTERISTICS AND CLINICAL COURSE

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Background. The development of a Non-Hodgkin's lymphoma (NHL) is one of the most serious complications in patients with autoimmune diseases (AD). Most of these lymphomas originate from B-cells and the extranodal mucosa associated lymphoid tissue (MALT) lymphoma is the most common subtype. Currently, it is unclear whether these lymphomas have a different clinical course or a different responsiveness to therapy compared to their counterparts in patients without autoimmune diseases.*Aims.* We have investigated the clinical characteristics of MALT lymphoma patients and the influence of AD on the clinical course as compared to controls without an underlying autoimmune condition.

Patients and Methods. We evaluated retrospectively 219 patients with histologically verified MALT lymphoma within a case-control study. In 134 cases, clinical and serologic data to judge the presence of AD were available. The epidemiologic (age), genetic (trisomy 3, trisomy 18, t(11;18), t(14;18) involving IGH/MALT1) and clinical data (site and extent of disease, relapse rate, time to relapse, monoclonal gammopathy) of these patients with autoimmune diseases were compared to those without AD. *Results.* In total, 63/134 patients (47%) suffered from a concurrent AD, with patients being significantly younger (59 vs 67 years, p= 0.003). Elevated autoimmune parameters without clinical significance and symptoms were found in 15.7% of patients. Patients with AD had significantly more extragastric lymphoma (p=0.011), but showed a comparable number of multifocal disease (p=0.7). Surprisingly, equal relapse rates (p=0.3) and a similar time to relapse were found in both groups (p=0.3, Figure 1).

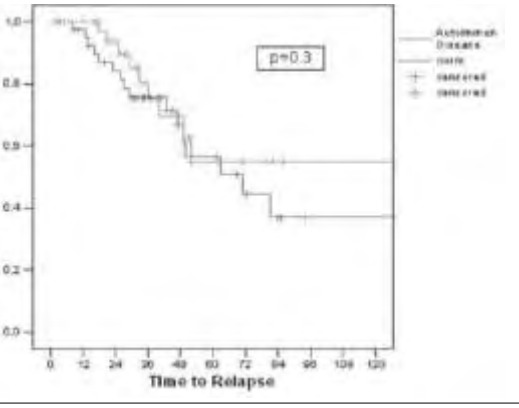

Figure 1. Time to relapse.

There was no difference with regard to genetic aberrations: trisomy

3 (p=0.057), trisomy 18 (p=0.8), t(11;18) (p=0.1) and t(14;18) (p=0.6). The presence of a monoclonal gammopathy/paraprotein production was evenly distributed (p=0.1) between both groups. *Summary/Conclusions.* This is the first study to suggest that the clinical course and the genetic aberrations of MALT lymphoma patients are not related to the presence or absence of autoimmune diseases. However, patients with autoimmune diseases develop MALT lymphomas at a significantly younger age. It was also shown that elevated autoimmune parameters were in fact associated with underlying AD in 85% of cases and were not merely a paraneoplastic phenomenon. Since a significant number of patients with MALT lymphoma suffer from an underlying AD, it is reasonable to determine autoimmune parameters on a routine basis in such patients and to search for the presence of an underlying AD.

0184

RITUXIMAB-M/VACOP-B COMBINED WITH RADIOTHERAPY IN PRIMARY MEDIASTINAL LARGE B CELL LYMPHOMA : A PROSPECTIVE ITALIAN INTERGROUP PHASE II STUDY

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Background. Weekly third generation regimens such as MACOP-B or VACOP-B (M/VACOP-B) in combination with involved-field radiotherapy (IFRT) seem to improve lymphoma-free survival of PMLBCL (Zinzani 2002, Todeschini 2004, Savage 2005). The superiority of R-CHOP over CHOP or CHOP-like regimens has been recently demonstrated in younger low risk NHL (Phreundschuh 2005). *Aims.* To evaluate the effectiveness and safety of Rituximab added to the standard M/VACOP-B regimens (R-M/VACOP-B) ±IFRT in PMLBCL. *Patients and Methods.* A total of 40 patients with PMLBCL were treated in six participating centers between February 2002 and July 2005. The median age was 38 years (range 17-54); 21/19 (53%) were females; 30 patients had stage II and 10 stage IV; 38 (95%) presented a bulky disease; LDH was increased in 26 (65%) and 21(53%) had a superior vena cava syndrome. According to the age-adjusted IPI score, 24 patients had an IPI = 0-1 and 16 an IPI = 2-3. All patients were treated with standard MACOP-B (30 patients) or VACOP-B (10 patients) regimens plus six cycles of Rituximab (375mg/m^2) given at weeks 3,5,7,9,11,13. Twenty-six patients (65%) received mediastinal IFRT at a median dose of 36 Gy. The response was evaluated in all patients after six cycles of chemo-immunotherapy, at the end of planned chemotherapy and after IFRT. *Results.* The response rate after six cycles of the planned R-M/VACOP-B regimen was CR/CRu = 20(50%), PR=19(47%) and NR=1(3%). Eight/40 patients received a second line therapy followed by HDT-ASCT (6/8 patients) because considered low responders (PR= 7 and NR=1). At the end of the chemo-immunotherapy program 28 patients witnessed a CR/CRu (70%) and 12 a PR (30%).Seven/12 PR patients obtained a CR/CRu following IFRT for an overall CR/CRu rate of 87% (35/40). After a median follow up of 13 months, the 2-year OS and PFS were 75% and 78%, respectively. No additional toxicities other than those related to the chemotherapy were observed during and after Rituximab infusion. *Conclusions.* R-M/VACOP-B are an active therapeutic regimens devoid of severe toxicity for the management of patients with PMLBCL. Further studies are required to demonstrate if the addition of Rituximab to front-line third generation regimens might overcome the need of more aggressive strategies, such as consolidation with IFRT or HDT-ASCT.

0185

ASSOCIATION OF REDUCED RELATIVE DOSE INTENSITY AND SURVIVAL IN LYMPHOMA PATIENTS RECEIVING CHOP-21 CHEMOTHERAPY

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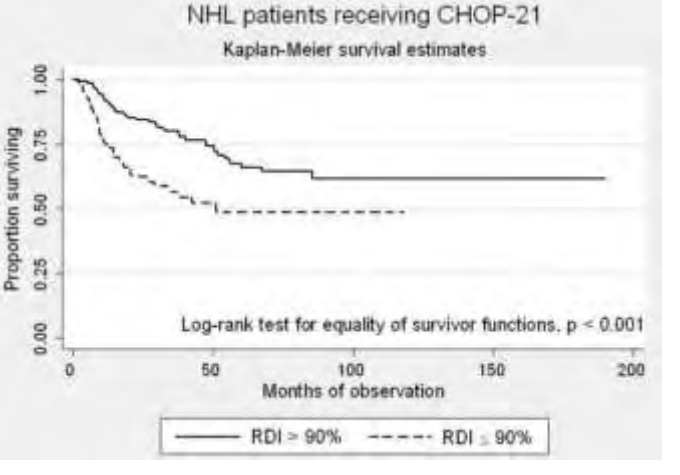
Background. Chemotherapy delivery in patients with non-Hodgkin lymphoma (NHL) is sometimes impaired by treatment side-effects. It remains unclear whether moderate reductions in relative (i.e., administered compared to planned) chemotherapy dose intensity (RDI) affect overall survival. *Aims.* To assess the relationship of reduced RDI and overall survival in NHL patients receiving CHOP chemotherapy with a cycle length of 21 days (CHOP-21). *Methods.* Retrospective audits of NHL patients were conducted in Belgium (Lymphodose '02) and the UK (Audit of Lymphoma Patients). Variables available from both datasets were merged into a dataset of individual observations, and definitions were harmonised, to allow for comparisons and combined analyses. RDI was averaged across anti-malignant drugs. Potential predictors of survival were assessed using extended Cox proportional hazards regression.



Results. The Belgian study included 211 NHL patients receiving CHOP-21 and the UK study included 78. Of these, 59% (Belgium) vs. 46% (UK) were female. Mean age (SD) at chemotherapy initiation was 63 (14) years (Belgium) vs. 55 (15) years (UK). Ann Arbour stages were distributed I 23%, II 34%, III 17%, IV 27% (Belgium) vs. I 19%, II 30%, III 27%, IV 24% (UK). During a mean observation time (SD) of 30 (17) months (Belgium) vs. 72 (38) months (UK), 31% vs. 35% of patients died. The Kaplan-Meier survivor functions differed (borderline p=0.06) and the proportion of survivors at 60 months was estimated to be 61% in Belgium vs. 67% in the UK. Mean RDI (SD) was 90% (17%) in Belgium vs. 94% (9%) in the UK (p=0.03). This difference was associated with a higher proportion of Belgian patients experiencing dose delays ≥7 days (38% vs. 29% in the UK; p=0.14). The proportion of patients receiving RDI ≤90%; RDI ≤85%; and RDI ≤80% was 30%; 25%; and 16% (Belgium) vs. 23%; 17%; 7% (UK). Kaplan-Meier plots showed reduced survival for those with reduced RDI, and the effect was strongest when the 90% cut-off (graph), or the 85% cut-off was used. Extended Cox regression using the combined dataset showed survival to be associated with age (hazard ratio 1.03 per year of age, 95% CI 1.01-1.05), RDI ≤≤90% (hazard ratio 1.8, CI 1.1-2.8), and stage of disease (hazard ratio 2.0 at treatment initiation, CI 1.5-2.9). The strength of the association with stage of disease decreased over time. *Summary/Conclusions.* This analysis confirms earlier reports that reduced

RDI may have a negative impact on survival in NHL patients receiving CHOP-21 chemotherapy. While further investigation is needed, delivering full chemotherapy dose intensity remains an important goal in this group of patients.

0186

THE EMERGING ROLE OF FDG PET/CT IN THE PRIMARY STAGING OF LYMPHOMA

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Background. The rapid adoption of FDG PET/CT scanning for imaging lymphoma has occurred in the absence of a large body of published experience of this imaging modality. *Aim.* To evaluate the role of PET/CT in primary staging of previously untreated Hodgkin (HL) or Non-Hodgkin lymphoma (NHL) and assess its impact on clinical management. *Method.* Over the period June 2003 to December 2005, we performed 1600 PET/CT scans for lymphoma. The primary staging cohort comprised 263 (16%) patients, (130 female, 133 male; mean age 52 years) from 64 referring specialists. All patients received 350 MBq FDG i.v with an uptake period of one hour; the study extended from the vertex to upper thighs; diabetic patients were not excluded. All scans were read without access to the histopathology or results of anatomical imaging. Histological diagnosis was HL (n=52) and NHL (n=211). DLBCL accounted for 41% and follicular lymphoma 34% of the NHL cohort. Referring doctors provided details of the clinical stage, results of other investigations and the management plan prior to the PET/CT. After the PET/CT they were asked for a revised clinical stage and management plan and the pre- and post-PET/CT staging and management plans were compared. Results were collated for 175 patients retrospectively and prospectively for 88. *Results.* There were 26 negative PET/CT scans: 23 were stage I patients where the sole primary site of disease had been resected, 2 had lesions beyond the resolution of the scanner (conjunctival MALT, cutaneous anaplastic large cell) and 1 scan was negative in a patient (HL) undergoing TPN with an elevated blood sugar level where the technical quality of the study was suboptimal. All were included in the analysis. Pre- and post-PET/CT staging was obtained in all patients. PET/CT altered staging in 105 (40%), 22 (42%) in HL and 83 (39%) in NHL. Staging was unchanged in 158. Up-staging was seen in 82 (78%) of those with change of stage (18 HL, 64 NHL). Pre - and post-PET/CT management plans were obtained in 91%. The 25 patients for whom we did not receive post PET/CT management plans were excluded from further analysis. Management was changed in 80 patients (34%) overall: 12 (27%) with HL and 68 (35%) with NHL; 44 patients were pre-PET/CT Stage 1, 14 Stage 2, 10 Stage 3 and 12 Stage 4. Management changes were made in 50% (36) of patients upstaged; 27% (39) with staging unchanged; and 28% (5) of those down-staged. The greatest impact on management occurred in upstaging patients with Stage I follicular lymphoma, of whom 71% had a change in management plan. *Conclusion.* Our data suggest that PET/CT detects a greater volume and extent of disease than was expected based on conventional staging. Further, this effect on staging significantly impacts on patient management. In addition, PET/CT also influences management decisions regardless of stage. Ongoing collation of disease specific data will further classify the influence of PET/CT scans in different histologies.

0187

BORTEZOMIB PLUS RITUXIMAB IN PATIENTS WITH INDOLENT NON-HODGKINS LYMPHOMA: A PHASE 2 STUDY

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Background. Bortezomib (VELCADE®), a first-in-class proteasome inhibitor, has demonstrated single-agent activity in non-Hodgkin's lymphoma (NHL) with response rates of 14-56%. Preclinical data with combined bortezomib and rituximab suggest increased activity with no overlapping toxicity. Weekly bortezomib is active in animal models for myeloma and is expected to be more convenient than the approved twice-weekly regimen. Weekly dosing was therefore studied in NHL. *Aims.* This randomised, phase 2 study investigated the response rate to bortezomib plus rituximab, weekly or twice-weekly, in patients with relapsed follicular lymphoma (FL) or marginal zone lymphoma (MZL). *Methods.* Eligibility criteria included CD20+ FL or MZL with measurable disease, and Karnofsky Performance Status ≥50% (ECOG 0-2). No prior bortezomib was allowed and patients who had received a prior regimen that included rituximab had to have responded and had a TTP of ≥4 months. Patients received bortezomib 1.3 mg/m^2 twice-weekly on days 1, 4, 8 and 11 of a 21-day cycle (Arm A) or bortezomib 1.6mg/m^2 weekly on days 1, 8, 15 and 22 of a 35-day cycle (Arm B) for up to 15 weeks (5 and 3 cycles in arms A and B, respectively). Starting from day 1, rituximab 375 mg/m^2 was administered weekly for 4 weeks in both arms. Response was evaluated by International Workshop Criteria. *Results.* 81 patients were enrolled between April 2004 and August 2005, and 74 (35 Arm A, 39 Arm B) were evaluable for response. Of these 81 patients, the majority had FL (80% Arm A, 93% Arm B); 76% of patients in Arm A received prior rituximab compared with 82% of patients in Arm B. Of the 81 treated patients, 11 (27%) in Arm A and 30 (75%) in Arm B completed all planned cycles. Median bortezomib dose received was 15.9 mg/m^2 (61% of the maximum expected) in Arm A, and 18.9 mg/m^2 (98% of the maximum expected) in Arm B. Overall response rate was 51% (2 CR + 2 CRu + 14 PR) in Arm A, and 54% (2 CR + 3 CRu + 16 PR) in Arm B. 11 patients had progressive disease (6 Arm A, 5 Arm B) and 1 patient died (Arm A). Response rates were similar in patients who previously received rituximab compared with the total study population. Median progression-free survival has not yet been reached (median follow-up 3.9 months). Treatment was well tolerated in both arms; grade ≥3 adverse events (AEs) were seen in 14 (54%) patients in Arm A and 6 (18%) patients in Arm B. The most common grade ≥3 AEs were gastrointestinal toxicities, neutropenia, thrombocytopenia and peripheral neuropathy. Serious AEs were seen in 11 (27%) patients in Arm A versus 6 (15%) patients in Arm B. *Conclusions.* Weekly and twice weekly bortezomib plus rituximab are active and well tolerated treatments for relapsed/refractory CD20+ indolent NHL. The more convenient weekly regimen appears to offer a similar response rate with less toxicity. The weekly regimen allowed delivery of a higher fraction of the intended dose, and a similar total cumulative dose, compared with the twice weekly regimen.

0188

CONSOLIDATION OF CHEMOTHERAPY RESPONSE IN MANTLE CELL LYMPHOMA PATIENTS WITH 90Y-IBRITUMOMAB TIUXETAN RADIOIMMUNOTHERAPY

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Background: MCL is an aggressive and prognostically unfavorable subtype of B-cell NHL, with a 5-yr survival rate <20%. Standard chemotherapy for MCL includes fludarabine, cyclophosphamide, and mitoxatrone (FCM). Addition of rituximab (R) to FCM increases the overall response rate (ORR) from 46% to 58%. Conventional non-myeloablative RIT has been unsuccessful in MCL patients, with a large tumor burden and no initial cytoreduction. *Aims:* The Polish Lymphoma Research Group Trial (PLRG MCL1) assessed whether 90Y-Zevalin would consolidate the response achieved from FCM-R and provide better ORRs and longer TTP. Methods: 22 MCL pts (stage III-IV) not suitable for SCTs were enrolled in 8 PLRG centers: 11 pts had MCL at diagnosis, 6 pts had a PR after first-line therapy of MCL, and 5 MCL pts were in first relapse. Tumor burden was reduced by treating all pts with a minimum of 3 to 6, three-wk cycles of FCM-R (375 mg/m^2) and staged after the third and subsequent cycles to assess tumor regression. Pts with a CR or PR, <25% bone marrow (BM) infiltration, <30 mm lymph node diameter, <12 cm spleen diameter, no massive extranodular involvement, PMNs >1500/µl, PLTs >100,000/µl, and no BM hypoplasia were entered into the 90Y-Zevalin consolidation step. They received 250 mg/m^2 of R and 1 wk later a second dose of R+90Y-Zevalin (11 or 15 MBq=0.3 or 0.4 mCi/Kg,

based on initial PLT count; max dose=32 mCi). ORR (CR+PR) was determined along with hematologic toxicity. Response is monitored at 6 wks, 3 mo, and 3-mo intervals for up to 2 yrs to assess TTP. *Results:* Following 90Y-Zevalin consolidation, 17 of 22 pts achieved a CR (no palpable lymph nodes or measurable masses on CT) and 13 of the 17 shifted from a PR after FCM?R to a CR. Three pts achieved a greater PR after 90Y-Zevalin. Of these 13 PR/CR pts, 11 are in CR 9-12 mo after 90Y-Zevalin, 1 pt progressed at 3 mo, and another progressed immediately despite 90Y-Zevalin. Overall projected 1-year PFS is >85%. Most pts experience a 7-10-fold in stem and progenitor cells' clonogenic capacity, preceding a drop of WBCs and PLTs 4'5 wks after 90Y-Zevalin (cytopenias lasting 5-7 wks). In 3 pts, the times for PMN >1000/µl were 7-12 wks and for PLT levels >50,000/µl were >20 wks and were transfusion dependent. No pts developed serious infections. Minimal, transitory (reversible by 2 wks) impairment of stromal cells was noted. Serum GM-CSF levels ≥ 2-fold at wk 4, and TPO and IL-3 ≥ 30% and 3-fold, respectively, at wk 2. EPO ≥ 3-fold during the first 4 wks and paralleled decreases in BFU-Es. *Conclusions:* 90Y-Zevalin consolidated the therapeutic benefit of FCM-R in 22 MCL pts. Fludarabine, a known radiosensitizer, reduces tumor burden before RIT and enhances the therapeutic benefit of non-myeloablative doses of 90Y-Zevalin, but may also ? the toxicity from RIT. Although 90Y-Zevalin has negative effects on stem and early progenitor cells, 90Y-Zevalin can be given safely after fludarabine-based therapy to high-risk MCL pts.

0189

MT103 (ANTI-CD19 X ANTI-CD3 BITE) INDUCES B CELL DEPLETION, CLEARANCE OF BONE MARROW INFILTRATION AND CLINICAL RESPONSES IN HEAVILY PRE-TREATED NHL PATIENTS: FIRST DATA FROM DOSE-ESCALATION STUDY MT103-104

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Background. MT103 is an anti-CD19/anti-CD3 bispecific single-chain antibody construct. Preclinical studies have shown that low picomolar concentrations of MT103 can redirect unstimulated human T cells against CD19-positive human B lymphoma and normal B cells leading to their efficient lysis. MT103 is further characterized by mounting a polyclonal T cell response, an activity at very low effector to target cell ratios, and an apparent independence of T cell costimulation. Corticosteroids were co-administered as anti-inflammatory agents. Initial findings have shown that MT103 has an estimated half-life of approximately two hours. Here we describe a continuous infusion regimen. *Aims.* The MT103-104 study was set up to explore the safety and tolerability of increasing doses of MT103 given as continuous infusion. Additional objectives include the assessment of MT103 continuous infusion PK profile, the collection of pharmacodynamic data and the observation of clinical efficacy. *Methods.* Patients with relapsed indolent NHL were included according to a classical 3+3 dose escalation design with initial MT103 doses of 0.5 mg/m^2/24h with initial steroid coverage. Safety and tolerability were assessed by CTC-AE criteria and dose escalation was only allowed after a data review committee (DRC) concluded safety of the previous dose with a DLT observation period of 14 days. Biological activity was monitored by investigating levels of systemic cytokines using specific ELISAs and by quantification and characterisation of peripheral immune cell subsets via FACS analysis. After 4 weeks of MT103 treatment, a control CT scan was performed. If patients were at least stable according to standardized Cheson criteria (reviewed by central radiology), an additional 4-week cycle of MT103 was offered to the patients. *Results.* As of today, 19 patients with a median number of 4 previous chemo-/immuno therapies have been included into MT103-104. During dose-escalation from DL1 (0.5 mg/m^2/24h) up to DL3 (5 mg/m^2/24h) no dose-limiting toxicity was observed, and AEs were generally moderate. At DL4 (5 mg/m^2/24h on the first day, 15 mg/m^2/24h as maintenance dose), 7 patients were treated with 2 patients receiving less than 14 days of treatment. One patient experienced elevation of liver enzymes up to CTC grade 3 after 2 weeks, which recurred upon re-administration of MT103; and 1 patient experienced confusion and disorientation on the second day of treatment. Depletion of circulating B (lymphoma) cells by end of the MT103 infusion was observed in 9 of 15 evaluable patients (with treatment for >2 weeks and B cells detectable in peripheral blood prior to MT103 infusion) with a dose-dependent increase in frequency that reached 100% depletion at DL4. At DL4, 3 of 7 patients had significant bone marrow (BM) infiltration (>10%) with 1 patient showing reduction of and 2 patients showing complete disappearance of lymphoma cells in BM. Best overall tumour response in the 14 evaluable patients (with treatment for >2 weeks and scanning of all involved areas) was 1 CR (at DL4), 2 PR (at DL4), 1 MR, 7 SD and 3 PD. *Summary.* These preliminary results observed in indolent NHL patients clearly indicate single agent biological and clinical activity of MT103. Further evaluation of dose and schedule is ongoing.

0190

PKC-BETA II EXPRESSION HAS PROGNOSTIC IMPACT IN NODAL DIFFUSE LARGE B-CELL LYMPHOMA

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Background. Recent studies of gene expression in diffuse large B-cell lymphomas (DLBCL) have identified gene signatures associated with germinal center or post-germinal center lymphocytes. These signatures have been shown to have prognostic significance in DLBCL, and to correlate with similar classifications based on immunohistochemical biomarkers. Protein kinase C β II (PKC-β II) was early identified as one such gene, and recent studies have suggested that its expression is associated with a poor prognosis. *Aim.* To determine the prognostic significance of the expression of PKC-β II in patients with nodal DLBCL. *Methods.* Patients with *de novo* nodal DLBCL treated in two hospitals were enrolled retrospectively. Inclusion criteria were treatment with anthracycline-based chemotherapy regimens, HIV-negative status, and tissue availability. Diagnosis was confirmed by a pathologic review board. Clinical data were obtained from the charts. The IPI was grouped in low-risk (0-2 factors) and high-risk (3-5 factors). Formalin-fixed, paraffin-embedded tissues were stained with a monoclonal antibody against PKC-β II protein (Sigma, P-3203). Cases with more than 10% stained large cells were considered positive. *Results.* A total of 125 patients were enrolled. The median follow-up was 5.3 years for the surviving patients. Data were available for IPI classification in 118 patients, and 83 patients were in the low-risk group. Forty-eight patients (38%) were positive for PKC-β II. There were no differences in LDH, age, B symptoms, Ann Arbor stage or IPI group according to the expression of PKC-β. More females than males were PKC-β II positive (54% vs 27%, p=0,003). Complete remission was obtained in 70%, and was not influenced by PKC-β II status (67% vs 71%). The 5-year event-free survival (EFS) was shorter in high-risk patients (14% vs. 58%, p<0.001) and in those with PKC-β II positivity (36% vs. 49%, p=0.0540). Only the IPI influenced the 5-year overall survival (18% vs. 70%, p<0.001). However, in low-risk patients, PKC-β II expression was related to a shorter 5-year OS (60% vs. 76%, p=0.03) and a shorter 5-year EFS (48% vs. 66%, p=0.014). In a Cox regression analysis for EFS, PKC-β II expression (hazard ratio = 1.6, p=0.04) and the IPI (HR=3.06, p<0,001) were independent poor prognostic factors. PKC-β II (HR=1.72, p=0.046) and the IPI (HR=5.16, p<0.001) were also independent poor prognostic factors for the OS. *Conclusion.* PKC-β II expression, along with the IPI, were associated with a shorter EFS and OS in patients with nodal DLBCL. PKC-β II identified a subgroup of patients, within the IPI low-risk group, who had a shorter OS.

0191

CIGARETTE SMOKING AND ALCOHOL CONSUMPTION AS DETERMINANTS OF SURVIVAL IN NON-HODGKINS LYMPHOMA: A POPULATION-BASED STUDY

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Background. The risk of non-Hodgkin's Lymphoma (NHL) seems to be enhanced by cigarette smoking and lowered by alcohol drinking. No study has ever focused on the role of these factors on survival from NHL. *Aims.* To assess whether cigarette smoking and alcohol drinking affect NHL survival *Methods.* A population-based prospective study on 1,138 Italian patients, diagnosed between 1991 and 1993, followed-up until 2002, was carried out. At diagnosis, clinical and socio-demographic data were recorded and lifestyle habits were assessed through a validated questionnaire. Survival analysis was performed with Kaplan-Meier

Methods. Hazard ratios (HR) were estimated by Cox regression. *Results.* The mean follow-up was 6.6 years (st.dev. 4.3). The mean survival time was 7.56 years (st.dev 0.155). At both univariate and multivariate analysis heavy cigarette smoking and alcohol drinking were associated with poor survival. Compared with those with a lower cumulative exposure to tobacco smoking, those who had smoked >31 pack-years had a worse survival (HR=1.60, 95%CI=1.18-2.18). Drinkers had a higher risk of death compared with non-drinkers (HR=1.41, 95%CI=1.10-1.81). Considering only those who had NHL as cause of death, the HR for the higher category of pack-years smoked, compared with the lowest, was 1.63 (95%CI=1.15-2.33) and for drinkers, compared with non-drinkers, it was 1.33 (95%CI=1.01-1.80). *Conclusions.* cigarette smoking and alcohol drinking may influence NHL survival.

0192

IBRITUMOMAB TIUXETAN COMBINED WITH HIGH-DOSE CHEMOTHERAPY AND AUTOLOGOUS STEM-CELL TRANSPLANTATION IN PATIENTS WITH CHEMO-REFRACTORY AGGRESSIVE NON-HODGKIN'S LYMPHOMA

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Background. High-dose chemotherapy and autologous stem-cell transplantation (SCT) have an established role in the treatment of patients with first chemo-sensitive relapse of aggressive lymphoma. However, autologous SCT has only limited success when performed in refractory or progressive stage of the disease and the expected 1-year progression-free survival (PFS) in this setting is less than 20%. The major cause of treatment failure is disease relapse. *Aims.* This study was designed to explore the safety and outcome following inclusion of Zevalin in the conditioning regimen given prior to autologous SCT. The primary endpoint was 1-year PFS. *Methods.* Patients were eligible for this study only if they had refractory lymphoma and a positive PET-CT prior to SCT. Rituximab 250 mg/m^2 followed by Zevalin 0.4 mCi/kg were given on day -14. Chemotherapy according to standard BEAM regimen was started on day -6. *Results.* The study included 22 patients, 14 man and 8 women, median age 55 years (range, 35-66). Histology was diffuse large cell (n=15), transformed follicular (n=6) and mantle cell lymphoma (n=1). Patients had active lymphoma at SCT, either primary refractory (n=12) or refractory relapse (n=10) and 12 patients had bulky disease at SCT. The median number of prior lines of therapy was 3 (range, 1-6). There were no early infusion reactions associated with Zevalin. Fifteen patients achieved CR (3 of them after additional radiotherapy given after SCT), 5 achieved PR and 2 died early after SCT from organ toxicities. With a median follow-up of 9 months (range, 1-21), 15 patients are alive and 7 have died. The estimated 1-year survival is 54% (28-81). Only 4 patients relapsed so far with a 1-year cumulative incidence of only 22% (9-53). As expected in this group of patients with refractory disease all relapses occurred within 4 months of SCT, such that despite the relative short follow-up relapse rate seems lower than expected. Two pts died of multi-organ toxicities and 2 of late occurring infections. The day 100 treatment related mortality was 9%. These rates of non-relapse mortality are expected in heavily pretreated patients with refractory lymphoma and there was no additional toxicity related to Zevalin. *Conclusions.* The inclusion of Zevalin in the conditioning regimens given prior to autologous SCT may reduce the risk of post SCT relapse and improve the poor outcome of patients with refractory lymphoma given SCT with standard regimens. This observation merits further study in larger comparative studies.

0193

CENTRAL NERVOUS SYSTEM INVOLVEMENT IN PATIENTS WITH NON-HODGKIN'S LYMPHOMA. INCIDENCE AND CLINICAL CHARACTERISTICS

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Purpose. The aim of the study was to evaluate the incidence of central nervous system infiltration in patients with Non-Hodgkin's lymphoma diagnosed according to the REAL/WHO classification and to describe the clinical features and treatment outcome. *Patients and Methods.* All patients diagnosed of a lymphoid neoplasm in our centre between May 1994 and May 2004 (n=2544) were included in our analysis. We identified all the cases with CNS infiltration at diagnosis or during the clinical course and excluded those who received intrathecal prophylaxis. We evaluated the incidence, clinical characteristics and response to treatment. *Results.* Forty (3.8%) patients with CNS infiltration were identified. Twenty one (52.5%) males, with a median age of 56 years (range 31-82 years). Ten (25%) patients presented CNS infiltration at diagnosis. Thirty patients (75%) developed CNS involvement during the course of the disease at a median time of 12 months (range 3.4-20.5m) from initial diagnosis. Four (10%) were HIV+. CNS infiltration by REAL/WHO subtypes is shown in the table below. Clinical and biological features at diagnosis were as follows: Stage IV: 31 (77.5%), extranodal involvement: 36 (90%), bone marrow infiltration: 15 (40.5%), bulky disease: 20 (50%), B symptoms: 14 (35%) and ECOG ≥2: 20 (50%). Response to treatment: CR 10 patients (27%), PR 5 (13.5%) and failure 22 (59.5%). Median overall survival was 2.26 months (range 0.72-3.8 months). *Conclusions.* The high incidence of CNS involvement in lymphoma patients' although previous CNS prophylaxis, makes us hypothesise that unknown factors could be associated to this phenomenon. Treatment response and survival of theses patients is poor.

Entity	*CNS Infiltration/Lymphoid Neoplasm*	*Incidence*
Diffuse large B cell lymphoma	26/368	7.1%
Follicular lymphoma	6/171	3.5%
Multiple myeloma	3/238	1.3%
Splenic marginal zone lymphoma	2/108	2.0%
Mamtle cell lymphoma	1/60	1.7%
Cutaneous T cell lymphoma	1/88	1.1%
Angiocentric lymphoma	1/10	10%
Total	**40/1043**	**3.8%**

0194

CLINICAL FEATURES OF THE WESTERN AND ASIAN FORMS OF INTRAVASCULAR LYMPHOMA (IVL) VARIES ACCORDING TO THE PRESENCE OF HEMOPHAGOCYTIC SYNDROME (HPS) AND NOT TO THE GEOGRAPHICAL AREA

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Background. Published data suggest the existence of some clinical differences between IVL patients diagnosed in Asian and Western countries. *Aim.* To explore potentially different clinical forms of IVL by comparing the clinical features of the largest cumulative series of IVL patients diagnosed in Western countries and three subgroups of IVL patients diagnosed in different Asian countries and published in the English literature. *Methods.* Clinical records and pathological material of 45 HIV-negative patients with IVL diagnosed in 8 Western Countries (Western-IVL) were reviewed. Clinical features of this series were compared with 282 previously reported cases of IVL diagnosed in Western countries (Western-IVL) and with 120 previously reported cases of IVL diagnosed in Japan (n=86) and other Asian Countries (n=34). Analysis was performed according to the presence of HPS. *Results.* HPS was absent in our patients; it was diagnosed in 5 (2%) previously reported cases of Western-IVL (p= 0.37), in 38 (44%) Japanese patients (p= 0.00001) and in 4 cases (12%) diagnosed in other Asian countries (p= 0.03). Analysis of differences in clinical presentation and laboratory findings included four subgroups: our 45 Western-IVL patients, 38 Japanese patients with IVL and HPS (J-HPS), 48 Japanese patients with IVL without HPS (J-IVL) and 30 patients with IVL without HPS diagnosed in Asian countries other than Japan (Eastern-IVL). Median age was very similar among studied subgroups, oscillating between 62 and 69 years, with a constant slight prevalence among males. As reported in the table, there were no significant differences in presenting symptoms, sites of disease or laboratory findings among Western-IVL, J-IVL and Eastern-IVL patients. Conversely, stage-IV disease, fever of unknown origin, involvement of liver, spleen, bone marrow or lung, fatigue, jaundice, thrombocytopenia, increased serum levels of hepatic enzymes as well as a concomitant extravascular lymphoma were significantly more common among the

38 J-HPS patients in comparison with the other groups. Conversely, skin and central nervous system involvement was significantly more rare in J-HPS patients. No significant differences were observed in terms of anemia, leucopenia, monoclonal component, and peripheral blood involvement. In patients treated with anthracycline-based chemotherapy (21 from our Western-IVL series and 27 from J-HPS series), complete remission rate was 52% and 58% (p= 0.92), with a 2-year overall survival of 45±11% and 22±8% (p= 0.04), respectively. *Conclusions.* The association between IVL and HPS is anecdotally diagnosed outside of Japan. IVL significantly varies in clinical features and laboratory findings according to the presence of HPS and not to the geographical area. Patients with IVL but without HPS diagnosed in Western countries, Japan and other Asian countries display similar characteristics and could be considered as forming part of a *classical form* of IVL. J-HPS patients display numerous clinical differences with respect to the classical form and could be considered as a *HPS-related variant* of IVL. When treated with anthracycline-based chemotherapy, both variants exhibits a worse prognosis, specially in HPS-related cases; thus, rendering advisable treatment intensification. An extensive phenotypic and molecular characterization is needed to confirm whether these clinical differences might reflect discordant biological entities within IVL.

	Western (n=45)	*J-HPS (n=38)*	*p*	*J-IVL (n=48)*	*p*	*Eastern (n=30)*	*p*
Fever	19 (42%)	34 (89%)	0.00001	20 (42%)	0.96	18 (60%)	0.13
Stage IV	34 (76%)	37 (97%)	0.004	45 (94%)	0.19	25 (83%)	0.42
Skin	17 (38%)	1 (3%)	0.0001	12 (25%)	0.18	7 (23%)	0.19
CNS	18 (40%)	8 (21%)	0.04	25 (45%)	0.24	11 (37%)	0.77
Liver	12 (27%)	25 (66%)	0.0004	15 (31%)	0.63	13 (43%)	0.13
Spleen	11 (24%)	29 (76%)	0.0001	10 (21%)	0.68	10 (33%)	0.40
Lymph n.	4 (9%)	2 (5%)	0.68	2 (4%)	0.43	9 (30%)	0.02
Lung	8 (18%)	14 (37%)	0.05	13 (27%)	0.28	15 (50%)	0.003
B. marrow	14 (31%)	28 (74%)	0.001	16 (33%)	0.82	4 (13%)	0.10
Thrombocyt	16 (36%)	28 (74%)	0.005	6/34 (18%)	0.007	9/25 (36%)	0.97
Hight LDH	29/34 (85%)	36 (95%)	0.17	31/33 (94%)	0.43	21/21 (100%)	0.14
High ALT	4 (9%)	10 (26%)	0.04	3 (6%)	0.71	8 (27%)	0.06
High bilirubin	2 (4%)	11 (29%)	0.004	0 (0%)	0.23	1 (3%)	1.00

0195

A PHASE II STUDY OF CYCLOPHOSPHAMIDE, VINCRISTINE, NON-PEGYLATED LIPOSOMAL DOXORUBICIN, AND PREDNISONE PLUS RITUXIMAB (R-COMP) IN ELDERLY PATIENTS WITH DIFFUSE LARGE B-CELL LYMPHOMA (DLBCL)

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Background. R-CHOP regimen has become the standard treatment for CD20+ diffuse large B-cell lymphoma (DLBCL). However, the majority of cases occur in elderly patients whose tolerance to immunochemotherapy is limited. Liposomal doxorubicin citrate (Myocet™) has an improved therapeutic index in comparison to standard doxorubicin, and may increase the tolerability of effective therapy in vulnerable populations. Patients and *Methods.* Up to eight cycles of the R-COMP regimen containing Myocet(tm) 50 mg/m^2, cyclophosphamide 750 mg/m^2, and vincristine 1.4 mg/m^2 (max. 2 mg) were administered on d1 every 3 weeks, plus rituximab 375 mg/m^2 (d3 C1, d1 thereafter) and prednisone 100 mg/d d1-5. Restaging was performed after 3 cycles and patients (pts) with an objective response received 5 additional cycles. A two stage Simon design was adopted. Complete response rates of 40% and 60% were defined in advance as the level of no interest (P0) and the level of interest (P1), respectively. Previously untreated elderly (>60 years) pts with CD20+ and stage IA bulky 'IV DLBCL were included. Pts with CNS involvement or VIH+ were excluded. *Results.* Between Oct 2002 and Apr 2005, 75 pts were registered. Fifty-nine pts were evaluable for efficacy. Reasons for exclusion from the evaluable population included patient ineligibility, patient not treated, early termination and failure to undergo disease re-evaluation according to the protocol total 16 patients. The median age was 71 years (range 60-83). At diagnosis, 56% of pts had an intermediate or high risk IPI score (≥ 2), 46% had B symptoms, 22% had Bulky disease > 10 cm in diameter, 70% had stage III or IV disease, 52% had increased LDH and 64% had extranodal disease. Median LVEF at baseline was 61% (range 50-89%). The mean number of cycles administered were 6.1 (range 1-8). 10% cycles (31% pts) were dose reduced and 5% cycles (26% pts) were delayed mainly due to haematological toxicity. The most important toxicity was haematological: 24% cycles (50% pts) had Grade (G) 3-4 neutropenia, 4% cycles (12% pts) febrile neutropenia, and < 1% cycles (3% pts) G 3-4 thrombocytopenia. Non haematological toxicity was low: 4% pts had G 3-4 constipation, and 3% pts had G 3-4 asthenia, mucositis, pyrexia, anorexia or alopecia. Median LVEF at last observation was 60% (range 40-79%). Four pts were withdrawn due to decrease in LVEF. One pt had acute pulmonary oedema (considered related to the drug in absence of other reasons). Sixty-six% pts (39) achieved a CR (28 CR and 11uCR) and 15% pts (9) had PR; 18% pts had no response (9 pts PD and 2 SD). *Conclusions.* R-COMP is an active regimen for the treatment of DLBCL in elderly pts producing a tolerable and predictable toxicity profile. Preliminary assessment indicates that the substitution of doxorubicin with Myocet™ may reduce cardiac toxicity of treatment.

0196

INDUCTION WITH FLUDARABINE, CYCLOPHOSPHAMIDE AND RITUXIMAB FOLLOWED BY RITUXIMAB MAINTENANCE IS A HIGHLY EFFECTIVE FRONT-LINE TREATMENT FOR PATIENTS WITH FOLLICULAR LYMPHOMA: RESULTS FROM A COOPERATIVE

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From October 2004 to January 2006, 75 newly diagnosed patients with grade 1,2 follicular lymphoma (FL) were included in a prospective study designed to evaluate clinical efficacy, evaluated as time to progression, after 6 cycles of a combined treatment with fludarabine (25 mg/m^2 x 3 days), cyclophosphamide (1 g/m^2/day1), and rituximab (375 mg/m^2/day1) (FCR) followed by maintenance treatment with rituximab (375 mg/m^2/week/ ×4 weeks/6 months/2 years). Clinical and molecular response rates as well as safety of this combination were also evaluated. We present results from the first 32 patients that completed at least 4 induction cycles. Median age was 57 years (30-74), 91% of the patients were in stages III-IV, 25% had bulky disease (>7 cm), and extranodal disease was presented in 85%. After 4 cycles all patients responded and 73% obtained a CR or uCR. Bcl2/IgH MBR and MCR rearrangements were studied by real time PCR and VDJ IgH, IgL Kappa VJ, K deleting and IgL Lambda VJ rearrangements were studied by Fluorescent PCR. Monoclonal population at diagnosis was identified in 62,5% of patients. 25 patients with a monoclonal population at diagnosis were studied after induction treatment and all patients obtained a complete molecular response. 13% of patients presented at least one serious adverse event being neutropenia the most common. Toxicity from the FCR regimen was observed specially among patients older than 60 years. Two cases of opportunistic infections were observed (CMV disease and cerebral toxoplasmosis). A profound and prolonged lymphopenia occurred in the majority of patients as well as some cases that developed a delayed neutropenia persisting during months after complete the induction treatment. We have observed that FCR regimen has a potent antitumoral activity in newly diagnosed patients with FL and all patients evaluable at molecular level have obtained a complete response; nevertheless profound immunosupression developed in some cases resulting in the development of opportunistic infections. Data from the 75 included patients will be actualized and presented at the time of the meeting.

0197

INTENSIVE CHEMOTHERAPY (HIGH-DOSE CHOP/ESHAP REGIMEN) FOLLOWED BY AUTOLOGOUS STEM-CELL TRANSPLANTATION IN PREVIOUSLY UNTREATED PATIENTS WITH PERIPHERAL T-CELL LYMPHOMA

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Background. the outcome of patients (pts) with PTCL receiving conventional therapy is dismal. Because of this, there is an increasing interest to investigate intensive treatments in these pts. *Aims.* to analyze the results in terms of toxicity, response and outcome, of a phase II trial that includes high-dose chemotherapy (CT) plus ASCT as first line treatment for pts with PTCL. *Methods.* forty pts (29M/11F; median age: 47 yrs) diagnosed with PTCL (excluding cutaneous and anaplastic ALK+), in stages II-IV and <65 yrs, who have finished the planned therapy, are the subject of this analysis. Pts received intensive CT (3 courses of high-dose CHOP [cyclophosphamide 2000 mg/m^2 day 1, adriamycin 90 mg/m^2 day 1, vincristine 2 mg day 1, prednisone 60 mg/m^2/day, days 1 to 5, mesnum 150% of cyclophosphamide dose, G-CSF 300 μg/day days 7 to 14], alternating with 3 courses of standard ESHAP). Responders (CR or PR) were submitted to ASCT. *Results.* twenty-three patients had a PTCL unspecified, nine angioimmunoblastic, two panniculitic and six other subtypes. Eleven pts (28%) presented with primary extranodal disease, 28 (70%) were in stage IV, and 14 (35%) had bone marrow involvement. Forty five percent of the pts had high/intermediate or high-risk IPI, whereas 49% were in the groups 3 or 4 according to the Italian Index for PTCL. Twenty seven pts (68%) received the planned 6 courses of CT. Response rate after CT was: CR, 19 cases (47.5%); PR, 4 (10%); failure, 17 (42.5%), including one pt who died because of sepsis. Hematological toxicity of CT mainly consisted of neutropenia (grades 3-4 in 87 and 62% after high-dose CHOP and ESHAP, respectively) and thrombocytopenia (grades 3-4 in 63 and 68%, respectively). Severe infection requiring hospitalization was observed in 38 and 15% of courses of high-dose CHOP and ESHAP, respectively. Only 16 of the 23 candidates (70% of all candidates and 40% of all pts) received ASCT due to the lack of stem-cell mobilization (3 cases), severe previous toxicity (2), early relapse (1) and pt decision (1). No differences in the outcome were seen among these 23 pts according to whether or not they eventually received ASCT. No major toxicity was observed after ASCT. Response after the whole treatment was: CR, 20 cases (50%), PR, 3 (8%), failure, 17 (42%). Two of 14 pts in CR and 2 pts in PR eventually progressed. Four-year failure-free survival (FFS) was 35% (95%CI: 14-46%), whereas 4-yr event-free survival for pts achieving CR was 63% (95%CI: 43-89%). Twenty-one pts have died during follow-up, with a 4-yr overall survival (OS) of 40% (95%CI: 21-55%). Most patients died because of PTCL progression, but 2 died in CR due to secondary leukemia and lung cancer, respectively. Both the IPI and the Italian Index were able to predict FFS and OS. *Conclusion.* in this series of patients with PTCL a relatively high CR rate was obtained with high-dose CHOP/ESHAP followed by ASCT. Toxicity was manageable. However, the prognosis of patients with PTCL, particularly of those not achieving CR, is still very unfavorable.

0198

THE ADDITION OF RITUXIMAB TO CHEMOTHERAPY MAY CHANGE PROGNOSTIC F ACTORS, INCLUDING TUMOR BCL-2 EXPRESSION, IN PATIENTS WITH DIFFUSE LARGE B CELL LYMPHOMA

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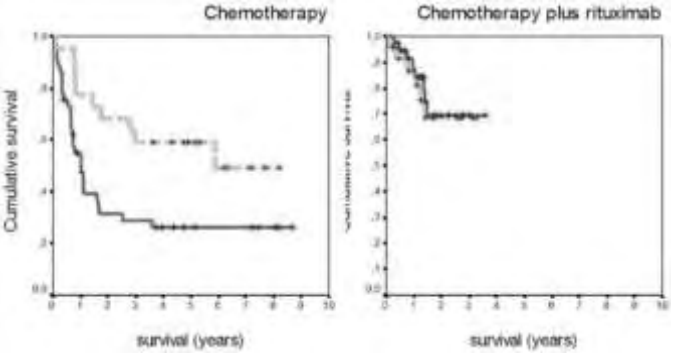
*Background and Aim.*Prognostic factors may change as a result of the introduction of better therapies. In this regard, the addition of rituximab to standard chemotherapy has highly improved the outcome of patients [pts] with diffuse large B-cell lymphoma [DLBCL]. The aim of the present study was to assess the outcome and prognostic factors, including bcl-2 tumor expression, in pts with DLBCL before and after the rituximab era. *Patients and Methods.* 131 pts (median age: 58 yrs; 77M/54F) diagnosed with DLBCL in a single institution between January 1997 and January 2005 were treated with CHOP-like regimens [CHOP] until Jan 2002 (N=60; median follow-up: 5.3 yrs) and with CHOP-like plus rituximab [R-CHOP] since that date (N=71; median follow-up: 1.7 yrs). The only criterion to include pts was the availability of histological material. Tumor bcl-2 expression was considered positive when superior to 25%. Median overall survival was 3.4 years. Main clinicobiological features were assessed for prognostic value. *Results.* Main initial characteristics, including IPI and bcl-2 expression, were similar (p>0.1) between patients receiving or not rituximab. CR rate was 50% and 71% for pts treated with CHOP and R-CHOP, respectively (p=0.01). 3-yr overall survival [OS] was of 36% (95%CI: 24-49) and 63% (95%CI: 50-76) for pts CHOP and R-CHOP, respectively (p=0.002). Age, performance status, LDH and IPI predicted OS both in the whole series and in the two treatment subgroups. Pts receiving CHOP with bcl-2 positive expression showed poorer OS than those bcl-2 negative (3-yr OS: 28 vs. 67%, respectively; p=0.02). On the contrary, no difference in terms of OS was observed according to bcl-2 expression in the group of pts treated with R-CHOP (figure). In the multivariate analysis, IPI (p=0.001), treatment (CHOP vs. R-CHOP) (p=0.01) and bcl-2 expression (p=0.02) were the most important variables predicting OS. These figures were similar in the group of pts treated with CHOP, whereas only IPI maintained prognostic interest in the subset of pts treated with R-CHOP. *Conclusion.* The addition of Rituximab to chemotherapy improves the outcome and changes prognostic factors, including the negative impact of Bcl-2 expression, in pts with DLBCL. More studies are warranted to assess changes in other biological predictive indicators.



Non-Hodgkin's Lymphoma - Clinical II

0199

CLINICAL OUTCOME OF LOW GRADE NON HODGKINS LYMPHOMA PATIENTS WITH BONE MARROW INVOLVEMENT DETECTED BY FLOW CYTOMETRY ALONE

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Background. BM involvement in low grade NHL patients results in stage IV clinical classification and has a negative impact on survival. The standard practice is morphologic examination of BM biopsy conducted at diagnosis. In many institutions flow cytometry (FC) is also routinely performed on BM aspirate samples accompanying respective biopsies. FC is believed to increase the sensitivity of the morphologic analysis by detecting occult lymphoma cells evading the pathologist's eyes. However, the prevalence of such finding and especially its clinical significance are largely unknown. In our institute bone marrow biopsies of NHL patients conducted after 1993 were routinely accompanied by bone marrow aspirates with FC analysis. *Aims.* We aimed to analyze the prevalence and clinical significance of BM FACS findings in patients with low grade NHL. *Methods.* We retrospectively reviewed the charts of all low grade NHL patients (small lymphocytic lymphoma, follicular small cleaved cell NHL, follicular mixed small and large cell, marginal zone B-cell lymphoma, mantle cell lymphoma and Waldenstrom macroglobulinemia) diagnosed or followed in the Hematology Unit between 1994 and 2004, who had undergone bone marrow biopsies and aspirates as a part of their diagnostic workup or before treatment. Flow cytometric results were considered positive if they showed either a ratio of immunoglobulin light chain expression of kappa:lambda >3:1 or lambda:kappa >2:1 in at least 2% of the gated population. Selected cells were analyzed by two or three color combinations: CD5 versus CD19, CD20 versus CD10, and kappa light chain versus lambda light chain occasionally with the addition of CD19 or CD20. *Results.* Lymphoma involving BM by morphology was found in the biopsies of 43 patients (61.4%) (BM+ group). Of the remaining 1 patient had inconclusive results and 26 patients had normal BM biopsies. Of these 27 patients the FC analysis was positive in 9 patients (BM-FC+ group) and negative in 18 (BM-FC- group). We could not compare the groups using FLIPI or IPI scores as a whole since both include the stage as one of the five summed parameters while BM involvement was different by definition between the groups. However, the groups had similar parameters that are prognostically important and are part of the FLIPI scoring system including age, hemoglobin and LDH levels and also the number of involved extranodal sites. Splenic involvement and number of involved nodal sites were higher in BM+ and BM-FC+ groups than in BM-FC- group. Significant differences in disease progression as indicated by time-to-treatment were observed. The median treatment-free period was shorter in the BM+ and BM-FC+ groups (1 month and 4 months, respectively) as compared with the BM-FC- group (31 months) (log rank test p=0.0195). BM-FC- patients had significantly longer survival time than BM+ and BM-FC+ groups. Median survival time was not reached for the BM-FC- patients while in the BM+ and BM-FC+ groups median survival times were 129 and 89 months respectively with no significant difference between them. (Log rank test=0.029 for the difference between BM-FC- and the two other groups). *Conclusions.* We conclude that the outcome of low grade NHL patients found to have malignant cells by FC analysis while their BM morphology is normal is the same as that of patients with histological involvement. This may imply that patients with localized disease who have bone marrow involvement by FC should be regarded as advanced stage disease.

0200

CHOP (DOXORUBICIN) CHEMOTHERAPY IS SUPERIOR TO CNOP (MITOXANTRONE) IN THE TREATMENT OF PATIENTS WITH AGGRESSIVE NON-HODGKIN LYMPHOMA (REVIEW)

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Introduction. Mitoxantrone (M), an anthracenedion, was introduced in the early/mid 1980s as a more tolerable alternative to anthracyclines. This agent has a broad anti-tumour activity including lymphoma with potentially less cardiotoxicity than doxorubicin (D), which may be of particular importance in the elderly patient population. However, an important issue is whether M is as efficacious as D in the treatment of NHL patients. *Methods.* Through search of several relevant databases and direct contacts with lymphoma investigators worldwide, we identified seven randomised studies of previously untreated patients comparing CHOP and CNOP chemotherapy in aggressive NHL. In this analysis we included five trials where (D; 50 mg/m^2) was compared with (M;10-12 mg/m^2; table) and the interval between chemotherapy courses was 3-4 weeks. Patients reported in the Pavlovsky article were included in the Bezwoda report, why analyses were performed with and without patients reported by Bezwoda *et al.* Odds ratios of complete remission (CR) and overall survival (OS) were pooled using a fixed effects model. *Results.* CHOP was significantly superior to CNOP with regard to both CR rate and OS (Figures 1-2).

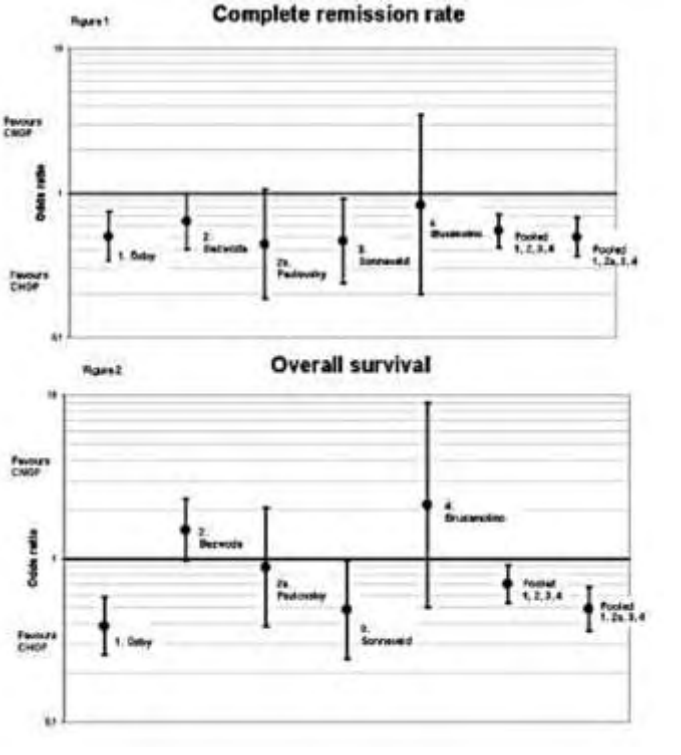

Table. Randomised trials comparing doxorubicin with mitoxantrone in untreated patiens with aggressive NHL.

Reference	*Regimen*	*Doses of doxorubicin/ mitoxantrone (mg/m^2)*	*Treatment interval (weeks)*	*Number of patients*	*Median age (years)*	*Number of patients ≥60 years*	*CR rate (%)*	*Overall survival (%)*
Osby	CHOP	50	3	205	71	205	60***	56*** (3 years)
et al., 2003	CHOP	10		203	70	203	43	33
Bezwoda	CHOP	50	3	164	55[2]	69	51[3]	40 n.s. (5 years)
et al., 1995	CHOP	10		161	54	70	40	50
Sonneveld	CHOP	50	4	72	70	72	49*	42* (3 years)
et al., 1995	CHOP	10		76	71	76	31	26
Pavlovsky	CHOP	50	3	44	NI	NI	70 n.s.	53 n.s. (4 years)
et al., 1992	CHOP	10		45	NI	NI	51 n.s.	50
Brusamolino	CHOP	50	3-4	20	57*[2]	NI	70 n.s.	56 n.s. (2 years)
et al., 1988	CHOP	12		15	47	NI	66	73

*p<0.05; **p<0.01; ***p<0.001; n.s.= Not significant; NI = No information; [1] Approximately (visual reading); [2]Mean; [3]p=0.05

Myelosuppression was not more severe using CHOP, rather the opposite. However, the incidence of gastrointestinal toxicity and alopecia was significantly lower in patients treated with CNOP. *Conclusion.* CHOP chemotherapy is more efficacious than CNOP at equitoxic (myelosuppression) doses leading to higher CR rates and improved survival.

0201

EARLY-MID TREATMENT C-REACTIVE PROTEIN LEVELS PREDICT TIME TO DISEASE PROGRESSION OR RELAPSE AS WELL AS OVERALL SURVIVAL IN AGGRESSIVE NON-HODGKIN'S LYMPHOMA

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Background. Higher pretreatment serum CRP levels in patients with aggressive non-Hodgkin's lymphoma (NHL) are associated with in a more aggressive histology, B-symptoms and a shorter overall survival (OS). In most patients who achieve complete remission (CR) at the end of therapy, serum CRP levels appear to return to normal range. In the light of an emerging role for early-mid treatment FDG-PET as an important prognostic indicator for progression free survival (PFS) and OS in NHL, we considered whether a simple parameter, such as early-mid treatment CRP, could also be a significant prognostic factor in this respect. *Aims.* To evaluate the possibility that wide ranged CRP could predict early response to treatment, time to progression or relapse and overall survival in aggressive NHL.

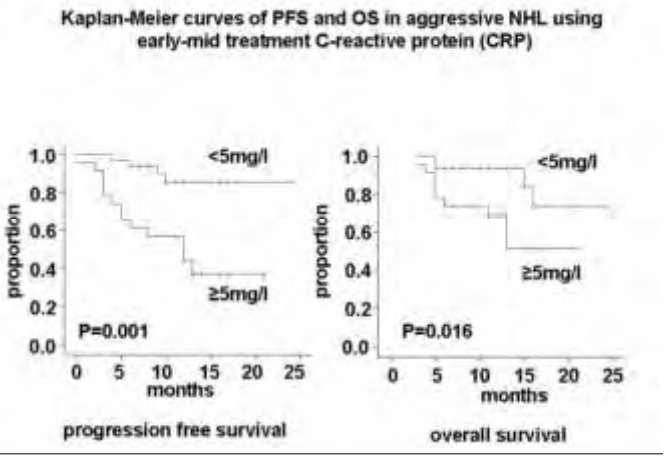

Figure 1. Kaplan-Meier curves of PFS and OS.

Patients and Methods. Serum CRP levels were monitored in fifty five patients with aggressive NHL (newly diagnosed and relapsed) at baseline and before receiving each of the next 3 chemotherapy cycles. The lowest value of the early mid-term CRP levels recorded was compared to the interim FDG-PET results, as well as to the clinical course and outcome. *Results.* At baseline, patients with aggressive NHL presenting with B-symptoms or bulky disease had higher pretreatment CRP levels compared to those recorded in asymptomatic patients and those without bulky disease (mean 90±71.9 mg/L Vs 37.7±41.9, p=0.0013 and mean 76.8±54.2 Vs 40.3±55.9 mg/L, p=0.04, respectively). Pretreatment CRP levels ≥20 mg/L were also associated with a shorter overall survival (p=0.029). During chemotherapy, the lowest value of early-mid treatment CRP levels significantly predicted the results of the interim FDG-PET (p=0.04 with a hazard ratio of 1.28). This implies that any increase of 1 mg/L in the serum CRP level enhances the risk of a positive FDG-PET scan by 12.8%. Moreover, patients who did not achieve an early-mid treatment CRP level of <5 mg/L, appear to have a shorter time to disease progression or relapse (p=0.001), and a reduced overall survival (p=0.016) (Figure 1). In multivariate analysis, both early-mid treatment CRP levels and interim FDG-PET findings significantly predicted PFS (p=0.02 and p=0.004, respectively), while OS was significantly predicted by the early-mid treatment CRP levels (p=0.016) and by the International Prognostic Index -IPI (p=0.03). *Conclusions.* The early-mid treatment serum CRP level is an important prognostic factor in aggressive NHL. Patients who do not achieve an early-mid treatment level of < 5 mg/L have faster disease progression or earlier relapse and also appear to have an inferior overall survival.

0202

BEAC OR BEAM CHEMOTHERAPY FOLLOWED BY AUTOLOGOUS STEM CELL TRANSPLANTATION IN NON-HODGKIN'S LYMPHOMA PATIENTS: COMPARATIVE ANALYSIS ON EFFICACY AND TOXICITY

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Background. Non-Hodgkin's lymphoma (NHL) is the major indication of high dose chemotherapy (HDC) followed by autologous stem cell transplantation (ASCT). However, little is known on the comparative efficacy and toxicity of various HDC regimens. *Aims.* This study aimed to compare the efficacy and toxicity of BEAC and BEAM regimen. *Methods.* Between April 1994 and February 2005, 97 NHL patients were received HDC with BEAC (N=69) or BEAM (N=28) followed by ASCT at Asan Medical Center. We matched one patient received BEAM with two patients received BEAC who has same International Prognostic Index (IPI). Thus total 84 patients (56 in BEAC group and 28 in BEAM group) were analyzed. *Results.* Of 84 patients, 55 (65.5%) were male, 29 (34.5%) were female and median age was 40.5 (15-65) years. Baseline characteristics such as age, sex, disease status at ASCT, histology, stage at ASCT, IPI were not different between two groups. Time to neutrophil engraftment (WBC >0.5×10^9/mm^3) was significantly longer in BEAC group (14.5days) than in BEAM group (11.0days, p=0.002). Total amount of RBC transfusion was more in BEAC group than in BEAM group (6.5 units vs. 3.7 units, p=0.037). Time to platelet engraftment (platelet >20 ×10^9/mm^3) was faster and total amount of platelet transfusion was less in BEAM group. Patients received BEAM had more frequent WHO grade ≥ 2 diarrhea than those received BEAC (46.4% vs. 19.6%, p=0.010). But, other clinically important toxicity such as mucositis, nausea/vomiting, bleeding were not different between two groups. In addition, neutropenic fever and documented infection were not different between two groups. Two year overall survival (OS) rate was 30% in BEAC group and 66% in BEAM group. Two year event free survival (EFS) rate was 34% in BEAC group and 61% in BEAM group. Both OS and EFS was significantly superior in BEAM group than in BEAC group (p=0.049, p=0.032, respectively). *Summary/Conclusions.* BEAM appears to be a superior HDC regimen in the aspect of OS and EFS than BEAC while regimen related toxicity is similar except more frequent diarrhea in BEAM.

0203

RAPID INFUSION OF RITUXIMAB WITH OR WITHOUT STEROID CONTAINING CHEMOTHERAPY. ONE YEAR EXPERIENCE IN A SINGLE CENTRE

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Background. Infusion-related toxicity is frequent after the administration of Rituximab despite the fact that strict guidelines have been recommended. Recently, a rapid rituximab infusion schedule in combination with a steroid containing chemotherapy regimen was well tolerated and safe. *Aim.* To asses the feasibility of a fast infusion of rituximab with or without steroid containing chemotherapy. *Methods.* Inclusion criteria: disease susceptible of treatment with rituximab and having been treated with a first infusion of rituximab according to the product monograph. Exclusion criteria: lymphocytosis > 5×10^9/L, toxicity grade 3/4 in the previous infusion of rituximab or dose > 375 mg/m^2. Schedule: First infusion of rituximab according to the product monograph; Further infusions over a total time of 90 minutes (20% in the first 30 minutes and the remaining 80% over 60 minutes). Premedication: acetaminophen and diphenhidramine, plus methylprednisolone in only those patients receiving steroid containing chemotherapy. *Results.* A total of 70 patients were treated for a total of 314 infusions. Patient characteristics: median age 64 yr (range 28-87), 47% males, DLBCL 36%, follicular 40%, mantle 6%, MALT 11%, other 7%. Number of rituximab infusions: 199 as treatment (combined or not with chemotherapy) and 115 as maintenance. Number of rituximab administrations with and without steroids: 123 and 191 infusions, respectively. Median time from previous rituximab infusion was 28 days (range 7-272). Sixteen rapid infusions were administered with an interval greater than 90 days from the previous standard infusions. This rapid rituximab administration schedule was very well tolerated. No grade 3/4 adverse events were seen. Three patients referred symptoms during rituximab infusion (grade 1) and all these reactions occurred in patients who did not receive premedication with steroids. *Conclusions.* Rituximab administration in a 90-minute infusion schedule is well tolerated and safe in this group of patients. This approach is beneficial, both in patients who are administered steroids and in patients who are not.

0204

RITUXIMAB PLUS CLADRIBINE OR CLADRIBINE AND CYCLOPHOSPHAMIDE IN HEAVILY PRETREATED PATIENTS WITH INDOLENT LYMPHOPROLIFERATIVE DISORDERS

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Background. Preclinical studies have shown synergistic or additive effects of rituximab combined with purine nucleoside analogs, fludarabine or cladribine (2-CdA). *Aim.*In this report we present the results of our study evaluating the feasibility, efficacy and toxicity of the combined regimens consisting of rituximab plus 2-CdA (RC regimen) or rituximab, 2-CdA and cyclophosphamide (RCC) in the treatment of patients with heavily pretreated indolent lymphoid malignancies. *Methods.* Between March 2001 and November 2005 54 adult patients with relapsed or refractory low grade non-Hodgkin lymphoma (LG-NHL) and B-cell chronic lymphocytic leukemia (CLL) were treated according to RC/RCC regimens. The RC protocol consisted of rituximab at a dose of 375 mg/m^2 i.v. on day 1 and 2-CdA given at a dose of 0.12 mg/kg/d on days 2 to 6. In RCC protocol rituximab was administered at a dose of 375 mg/m^2 on day 1, 2-CdA 0.12 mg/m^2 days 2 to 4 and cyclophosphamide at a dose 650 mg/m^2 i.v. on days 2 to 4. The cycles were repeated every 28 days or longer if severe myelosuppression occurred. Guidelines for response were those developed by the NCI- sponsored Working Group. *Results.* Fifty four patients, 32 patients with B-CLL and 22 with LG-NHL entered the study and all of them were eligible. Thirty three patients (61.1%) were recurrent after prior therapy and 21 (38.9%) had refractory disease. All patients received 3 or more cycles of chemotherapy before RC/RCC treatment. Thirty-one patients were treated with RC regimen and 23 with RCC regimen. The RC/RCC courses were repeated at 4 week intervals or longer if severe myelosuppression occurred. One hundred fifty six cycles of RC/RCC with median of 3 cycles per patient were administered (range 1-5 cycles). Six patients (11.1%), 2 with B-CLL and 4 with LG-NHL, achieved a complete response (CR). Thirty two patients (59.25%), including 23 with B-CLL and 9 with LG-NHL, had a partial response (PR). Overall response rate (OR) was 70.4% in the whole group, from 59.1% in LG-NHL to 78.1% in B-CLL patients. The median failure-free survival (FFS) of responders was 10.5 months. Hypersensitivity to RIT was the major toxicity of RC/RCC regimens, and occurred in 14 patients (25.9%), mostly during the first infusion of RIT. Severe neutropenia (grade III-IV) was seen in 5 patients (9.25%). Eight (14.8%) episodes of grade III-IV infections were observed. One patients died from severe pneumonia complicated with septic shock after second cycle of RCC regimen. Severe thrombocytopenia (grade III-IV) occurred in 4 patients (7.4%). *Conclusion.* RC and RCC regimens are highly effective and well tolerated modalities of treatment in heavily pre-treated patients with indolent lymphoproliferative disorders.

0205

A RETROSPECTIVE STUDY TO ASSESS RELATIVE DOSE INTENSITIES IN PATIENTS WITH LYMPHOMA IN CENTRAL EUROPEAN COUNTRIES

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Background. Maintaining chemotherapy dose intensity is important for the successful treatment of cancer patients. However, neutropenia and its complications are major dose-limiting factors. Data on chemotherapy-related reductions in dose intensities for lymphoma patients from Central European (CE) countries remain sparse. *Aim.*To assess the relative dose intensities in patients with Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL) in CE countries. *Methods.* Chemotherapy treatment data from 1995 to 2004 were retrospectively collected from 484 patients undergoing chemotherapy treatment for lymphoma from 24 centres in 4 CE countries: Czech Republic (26%), Hungary (13%), Poland (44%) and Slovakia (17%). For this sub-analysis, 310 patients who received either doxorubicin, vinblastine, bleomycin and dacarbazine (ABVD) treatment for HL (117 patients) or cyclophosphamide, doxorubicin, vincristine and prednisone ± rituximab every 21 days (CHOP21±R) for NHL (193 patients) were considered. *Results.* Of 116 HL patients with full data records (median age 29 years), 112 (96%) had *classical* disease and 4 (3%) had lymphocyte-predominant HL; for 189 NHL patients with full records (median age 56 years), 179 (95%) were B-cell and 10 (5%) were T-cell. AVBD was administered to 100% of the 117 HL patients selected for this study and CHOP21±R was administered to the 193 NHL patients, of which 39 patients (20%) also received rituximab. Dose delays ≥7 days were observed in 271 out of 1583 cycles (17%; HL: 110 of 648-17%; NHL: 161 of 935 - 17%). Overall, 221 patients (72% of 306 considered for this analysis) experienced at least one dose delay during their treatment. This corresponds to 95 of 117 HL-AVBD patients and 126 of 189 NHL-CHOP21±R patients (i.e. 81% and 67% respectively). One hundred and forty-three patients (47% of 305 patients) experienced a dose reduction of ≥15% in at least one cycle, of which 90 patients (30% of 305) received ≥15% reduction in their overall dose. Dose reduction of ≥ 15% in any cycle occurred in 61 HL-AVBD patients (52% of 117) and in 82 NHL-CHOP21±R patients (44% of 188), with 51 HL-AVBD and 39 NHL-CHOP21±R patients receiving ≥15% reduction in their overall dose (44% and 21% respectively). The relative total dose intensity (RTDI, see Table 1) at the end of treatment was as follows: 55.6% of HL-AVBD patients received ≥ 85% RTDI and 39.3% received ≥90% RTDI; 73.4% of NHL-CHOP21±R patients received ≥85% RTDI and 59% received ≥90% RTDI. G-CSF was administered in 71 of 765 cycles of AVBD (9.3%) chemotherapy and in 73 of 1124 cycles of NHL-CHOP21±R (6.5%). There were 48 unplanned hospitalisations in 30 patients (5 HD-AVBD and 25 NHL-CHOP21±R); 21 hospitalisations were neutropenia-related. *Summary/Conclusions.* The reduction of RTDI, and its associated problems, in lymphoma patients receiving chemotherapy is a major concern. The data observed in CE countries are similar to US centres (Lyman *et al.* JCO 2004; 22: 4302-4311). Further analysis of these data will enable a better understanding of the implications of reduced RTDI in lymphoma and help to identify those patients who require preventative treatment.

Table 1. RTDI at end of treatment.

	<75%		≤75<85%≥		≥85<95%		≥95%		≥90%		
Patient group	*N*	*(%)*	*N*	*(%)*	*N*	*(%)*	*N*	*(%)*	*N*	*(%)*	*Total patients**
HL-AVBD	32	(27.35)	20	(17.09)	38	(32.48)	27	(23.08)	46	(39.3)	117
NHL-CHOP21	33	(17.55)	17	(9.04)	69	(36.70)	69	(36.70)	111	(59.0)	188

*Total number of patients with data available for this analysis.

0206

COMPARISON BETWEEN 2-DEOXY-2-[18F]FLUORO-D-GLUCOSE POSITRON EMISSION AND COMPUTER TOMOGRAPHY FOR STAGING OF PATIENTS WITH HODGKINS LYMPHOMA

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Backgroud. Accurate staging in lymphoma patients (pts) has an important role in the treatment and allows minimization of toxic therapies, such as extended field radiation or overly aggressive chemotherapy. Particularly in HL a tailored therapy decrease the risk of secondary malignancies which exceeds 10% in several historical series in patients with early stage disease. Anatomic imaging modalities lack sensitivity and specificity because the definition of lymph node involvement is based on size criteria. During the last decade FDG-PET has been introduced for noninvasive staging of lymphoma. *Methods.* Herein we propose a prospective multicentric study with the aim to assess the impact of FDG-PET on the staging of pts with diagnosis of HL. A total of 186 consecutive pts coming from six Italian hematological Institutions underwent a FDG-PET scan in addition to conventional staging procedures, which include physical examination, laboratory data, bone marrow biopsy and imaging of the neck, thorax, abdomen and pelvis using CT scan. In general the adjunctive informations from PET did not influence the therapuetic options in use at a given centre at a particular time. *Results.* Pts characteristics were the following: 98 male and 88 female, 140 (75%) with diagnosis of nodular sclerosis classical HL, 28 (15%) mixed cellularity classical HL, 11 (6%) lymphocyte-rich classical HL, 2 (1%) lympho-

cyte-depleted classical HL and 5 (3%) non specified HL. At clinical and instrumental standard staging 11 (6%) pts were stage I, 112 (60%) stage II, 42 (22%) stage III and 21 (12%) stage IV. FDG-PET and CT were concordant in 156 out 186 pts (84%). FDG-PET allowed to identify in 38 out 156 concordant stage more nodal (32 pts) or extranodal (6 pts: two bone, two spleen, two liver and spleen) involvement in comparison with CT imaging. In eight out 156 (5%) concordant stage CT showed one more involved site in comparison with FDG-PET. FDG-PET results suggested an upstage in 27 pts (15%) and a downstage in 3 pts (2%). Fourteen pts (8%) with localized disease (I-II) at standard staging changed in an advanced stage as a result of the FDG-PET scan: three pts shifted from II to IV stage and ten pts from II to III stage and one from I to III stage. The information provided by FDG-PET led to a change in the therapeutic options in particular for pts which shifted from localized to advanced stages 10/14 (74%). *Conclusions.* Our data confirm that conventional staging system has an high sensibility nevertheless in this large cohort of pts FDG-PET is a relevant noninvasive method that supplements conventional procedures and should therefore be used routinely to stage HL particularly in pts with early stage, where a change in staging will modify disease management.

0207

MARKED ACTIVITY OF BORTEZOMIB, RITUXIMAB, AND DEXAMETHASON (BORID) IN HEAVILY PRETREATED PATIENTS WITH MANTLE CELL LYMPHOMA

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Background. Bortezomib (B) belongs to a new class of anti-cancer agents, the proteasome inhibitors, and has documented activity in multiple myeloma and mantle cell lymphoma (MCL). Preclinical studies suggest that B has synergistic activity with rituximab (R), which provides a rationale for the exploration of treatment combinations. *Aims.* We have initiated a phase II study in relapsed/chemotherapy refractory MCL to evaluate the activity and safety of B in combination with R and dexamethasone (BORID). *Patients and Methods.* A treatment cycle consists of B at 1.3 mg/m^2 administered on days 1, 4, 8, and 11, R at 375 mg/m^2 administered on day 1, and dexamethasone 40 mg orally on days 1 to 4. Cycles are repeated every 3 weeks for a total of 6 treatment cycles. Patients (pts) with progressive MCL after at least one prior line of therapy (including CHOP or a CHOP-like regimen) are eligible. *Results.* Up to now, we have enrolled 11 pts (median age, 67 years; range, 48 to 75 years) after a median of 3 lines of prior therapies (range, 1 to 6) including R in 9 pts, high-dose therapy in 4 pts, and thalidomide in 5 pts. Median time between start of frontline therapy and study inclusion was 42 months (range, 11 to 98 months). Severe adverse events (> grade II) included infections (herpes zoster in 2 pts, bacterial pneumonia, mucosal candidiasis), peripheral neuropathy (3 pts), fatigue (2 pts) and vasculitic skin infiltrates in 3 pts. Thrombopenia (< 50 g/L) occured in 2 pts. All adverse events were managable by standard means of supportive care and prolongation of the treatment interval between cycles. Of 9 pts evaluable for efficacy, 8 have achieved a response (3 CR, 4 PR), and 1 pt experienced stable disease. Pts in CR were also negative for disease activity by PET scanning. Skin infiltrates (histologically proven T-cell infiltrates) preceded achievement of CR in 2 pts. Among 7 pts with follow-up beyond 6 months, 2 pts have relapsed (progression-free survival 9 and 11 months, respectively), and 5 pts are still progression-free at 12, 11, 11, 7, and 6 months, respectively, after treatment initiation. Recruitment of patients is ongoing, and updated results will be presented. *Conclusions.* Data obtained thus far indicate that BORID has promising activitiy and managable toxicity in patients with heavily pretreated MCL, and development of a vasculitic rash may be an early indicator of a favorable response.

0208

CLINICAL AND CYTOGENETIC CHARACTERISTICS OF HIGH-GRADE NON-HODGKIN LYMPHOMA (HGNHL) WITH A COMBINATION OF T(14;18) TRANSLOCATION AND C-MYC REARRANGEMENT: A VERY AGGRESSIVE ENTITY WITH A DISMAL PROGNOSIS.

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Diffuse Large B cell lymphoma (DLBCL) is an heterogeneous entity with various clinical, cytological, cytogenetic and molecular features. In this single center retrospective analysis, we report the results of a cohort of 14 patients treated from 1997 to 2005 with a diagnostic of DLBCL or 'Burkitt-like' lymphoma characterized by a tandem t(14;18) translocation and c-myc rearrangement. Patients were 9 males, 5 females with a median age of 52 years (36-73). At the time of diagnosis, all patients presented with poor clinical and biological features: B symptoms in 78,5%, ECOG PS ≥ 3 in 64%, elevated LDH in 100%, stage IV in 100% (bone marrow involvement in 86% and CNS involvement in 36%), International Prognostic Index (IPI) ≥ 3 in 71,4% of the cases, respectively. Histological analysis and immunophenotyping performed on nodal tissue biopsy and/or bone marrow tumor cells showed a DLBCL in 9 and a 'Burkitt-like' in 4 patients, respectively. Cytogenetic analysis (conventional cytogenetics and FISH analysis) showed in all cases the combination of t(14;18) translocation and c-myc rearrangement. All patients were treated with chemotherapy regimens (R-CHOP (n=8) or High-dose CHOP (n=6)), and 5 could receive subsequent high-dose front-line therapy with autologous (n=3) or allogenic stem cell transplantation (n=2). Most patients (12/14=86%) initially responded to induction chemotherapy but disease response was dramatically short, precluding a planned stem cell transplantation in most cases. Despite salvage chemotherapy, all patients, even those who could receive early stem cell transplantation, progressed and the median overall survival from diagnosis is 4 months (1-10). In conclusion, DLBCL with a tandem t(14;18) translocation and c-myc rearrangement is a very aggressive entity with rapid progressive disease. Innovative strategies are warranted in this subgroup of patients.

0209

TRANSPLANT REGIMENS FOR HIGH-RISK MANTLE CELL LYMPHOMA: THE EFFECT OF INCORPORATING IBRITUMOMAB TIUXETAN RADIOIMMUNOTHERAPY ON RELAPSE RATE

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Background. High-dose therapy with autologous stem cell transplantation (ASCT) is an effective salvage therapy for patients with relapsed or refractory non-Hodgkin's lymphoma (NHL), but relapse rates remain high, especially in those with mantle cell lymphoma (MCL). *Aims.* One strategy for reducing relapse rates is to incorporate new active agents into high-dose regimens. With the activity of single-agent yttrium-90 (90Y) ibritumomab tiuxetan (Zevalin®) having been demonstrated in relapsed MCL, two clinical trials were initiated at the City of Hope to assess the safety and efficacy of incorporating ibritumomab tiuxetan radioimmunotherapy into conditioning regimens of patients with poor-risk or relapsed NHL. *Methods.* Patients <60 years and those without prior exposure to radiation therapy were accrued to a phase I/II dose escalation trial of high-dose 90Y ibritumomab tiuxetan (maximum target dose of 1000 cGy to normal organs) with cyclophosphamide 100 mg/kg and etoposide 60 mg/kg; patients >60 years and those with a prior history of radiation therapy were accrued to phase I/II trial of standard-dose 90Y ibritumomab tiuxetan (0.4 mCi/kg) followed by high-dose BEAM (BCNU 300 mg/m^2, cytarabine 800 mg/m^2, etoposide 800 mg/m^2, melphalan 140 mg/m^2). *Results.* Between June 2000 and January 2005, 8 and 10 patients were enrolled to the escalated-dose and 90Y BEAM trials, respectively. The median age of patients was 58 years (range, 44-72). Disease status at ASCT included 9 in first complete response (CR) (high or high intermediate IPI score), 4 in first partial response, 4 in first relapse, 1 in second CR. Nine patients (50%) had received HyperCVAD chemotherapy and 10 (56%) had received rituximab prior to ASCT. All patients had advanced-stage disease, including 13 with stage IV disease. The median 90Y dose administered was 40 mCi (range, 27-100). Treatment with either regimen was well tolerated. Engraftment to absolute neutrophil count > 500 cells/mm^3 occurred at a median of 10 days (range, 9-26). Five patients (28%) had reversible grade 3 pulmonary toxicity, including steroid responsive pneumonitis (n = 4) and sepsis-related acute respiratory distress (n = 1). Five patients relapsed and 3 of them subsequently died of progressive disease. PCR testing is available on 14 patients post-ASCT and all remain PCR negative for t11.14 in either bone marrow or peripheral blood. At a median follow-up of 24 months (range, 5-66), the estimated 2-year overall survival and disease-free survival are 81% (confidence interval [CI]: 65'91) and 65% (CI: 46-80), respectively. The relapse rate at 2 years is estimated at 24% (CI: 12-40). The dose of

90Y ibritumomab tiuxetan did not correlate with the risk of relapse as determined by univariate analysis. *Summary/Conclusions.* 90Y ibritumomab tiuxetan may be safely incorporated into conditioning regimens prior to ASCT, even in patients >60 years. Late relapses were uncommon, which suggests this approach may lead to durable remissions in MCL.

0210

GASTRECTOMY PLUS CHEMOTHERAPY VS. CHEMOTHERAPY ALONE IN GASTRIC DIFFUSE LARGE B-CELL LYMPHOMA OF EARLY STAGE

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Background. Stomach represents the most common site of primary extranodal diffuse large B-cell lymphoma (DLBCL). The ideal therapeutic strategy in gastric DLBCL remains controversial. Gastrectomy prior to chemotherapy is favored by some, while others prefer sole administration of chemotherapy. *Aim.* To evaluate and compare these two therapeutic strategies, gastrectomy plus chemotherapy and chemotherapy alone, in gastric DLBCL of early stage in the context of a retrospective study. *Methods.* Between 1979 and 2003, 78 patients with gastric DLBCL of early stage (I-II, no X) were diagnosed and treated in our department. Patients were divided in group A that comprised 46 (59%) patients, who underwent total gastrectomy prior to chemotherapy and group B that consisted of 32 (41%) patients, who received chemotherapy alone. Chemotherapy in both groups included CHOP and CHOP-like regimens. Median number of chemotherapy cycles administered in groups A and B was 6 (3-9) and 6 (2-8) respectively ($p>0.05$). Rituximab was also administered in 14 (30.4%) patients of group A and in 12 (37.5%) patients of group B ($p>0.05$). Five (11%) patients of group A and 2 (6.3%) of group B received additionally radiation therapy ($p>0.05$). The characteristics of our patients (gender, age, stage, IPI, presence of B symptoms and extranodal involvement other than primary), as well as response rates, were compared between the two groups using chi-square tests. Disease-free survival (DFS), overall survival (OS) and failure-free survival (FFS) were estimated according to the Kaplan-Meier method. Differences in survival rates were assessed using the log-rank test. *Results.* Median follow-up time for patients in groups A and B was 70 (3-270) and 46 (2-155) months respectively. On an intention-to-treat basis, the complete response rate was 91.3% for group A and 87.5% for group B ($p > 0.05$). DFS, OS and FFS rates at 4 years in groups A and B were 89.1% and 92.9%, 80.2% and 81.5%, 75.3% and 76.3% respectively ($p>0.05$). *Conclusion.* Gastrectomy plus chemotherapy failed to prove its superiority as treatment for gastric DLBCL of early stage in our study. Similar response and survival rates were achieved with chemotherapy alone, saving at the same time the patient from the morbid impact of gastrectomy on quality of life.

0211

PREDICTORS OF SURVIVAL IN ELDERLY PATIENTS WITH AGGRESSIVE LYMPHOMA. A LONG-TERM FOLLOW-UP OF A RANDOMISED TRIAL

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Background. We previously reported the results of a study in elderly patients (>60 years) with aggressive non-Hodgkin lymphoma (NHL) randomizing patients to receive CHOP (doxorubicin 50 mg/m^2) or CNOP (mitoxantrone 10 mg/m^2) with or without G-CSF (5 µg/kg from day 2 until day 10-14 of each cycle every 3 weeks; 8 cycles; Blood 2003;101: 3840). In that analysis 35% of patients were alive after a median follow-up time of 57 months. The main findings were 1) patients receiving CHOP fared better than those given CNOP chemotherapy and 2) the addition of G-CSF reduced the incidence of severe granulocytopenia and infections. We now report long-term follow up data with special emphasis on predictors of survival. *Methods.* The study included 455 previously untreated patients (median age 71 years; range 60-86 years) with stage II to IV aggressive NHL. Forty-seven patients previously hospitalized for class I to II congestive heart failure were randomized to receive CNOP with or without G-CSF (not included in the CHOP versus CNOP analysis). *Results.* After a median follow-up time of 115 months (18-151 months) 19% (88/455) of patients were alive. In univariate analysis CNOP treatment ($p<0.001$;figure), increasing age ($p<0.001$), poor performance status ($p<0.001$), high LDH ($p<0.001$), advanced stage ($p=0.035$), and the presence of more than one extranodal disease manifestation $p=0.045$) negatively influenced overall survival from diagnosis. Gender ($p=0.156$), presence of B symptoms ($p=0.079$), bulky disease ($p=0.085$), and treatment with G-CSF ($p=0.094$) did not significantly affect overall survival. In multivariate analysis, all factors significant in univariate analysis except extranodal disease, remained significant and independent predictors of survival. *Conclusion.* At long-term follow-up of this large multicenter randomised study of elderly with aggressive NHL the projected 10-year survival of CHOP treated patients was in excess of 20%. Increasing age, poor performance status, high LDH and advanced stage independently predicted a poor survival.

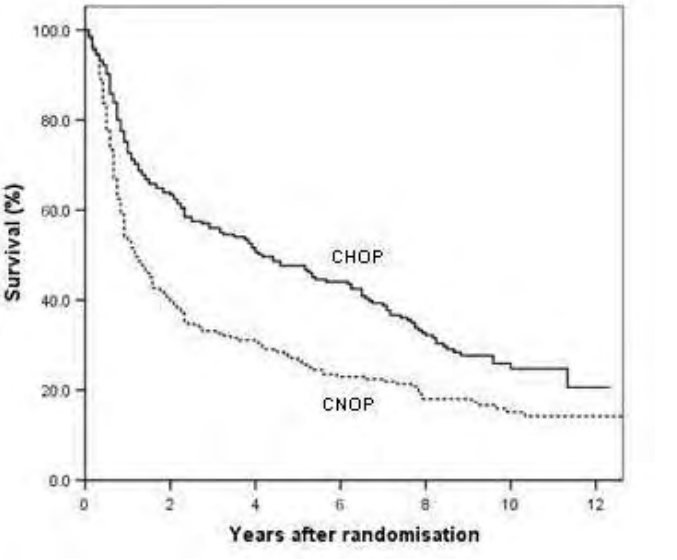

Figure 1. Survival according to type of chemotherapy.

0212

UPDATE REPORT ON 78 PATIENTS WITH POST TRANSPLANT LYMPHOPROLIFERATIVE DISORDERS FOLLOWED BY A SINGLE CENTER

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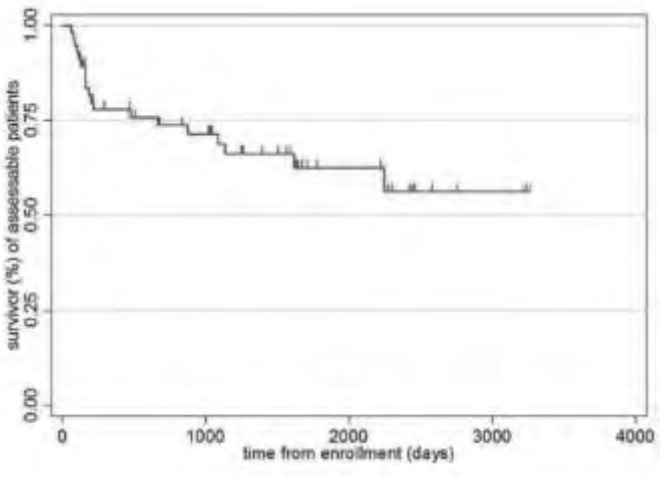
Background. Post transplant lymphoproliferative disorders (PTLDs) are a well recognized complication after solid organ transplantation, related to the chronic immunosuppressive regimen. The wide spectrum of histological features, clinical variability and high therapy-related toxicity make management of PTLD patients difficult. *Aims.* This abstract provides an update of clinical and pathological data of PTLD patients, followed at our Center between 1989 and 2006. *Methods.* Our study included 78 patients with a diagnosis of PTLD in solid organ transplant recipients (36 heart, 23 liver, 17 kidney and 2 lung). Morphological classification was made according to WHO criteria. In 72/78 patients tumour EBV status was tested by *in situ* hybridization for EBV encoded RNA (EBER). *Results.* It was not possible to assign 2/78 cases to any histological category because of inadequate specimens; 6/76 (8%) evaluable patients were classified as having Plasmacytic Hyperplasia (PH), 13/76 (17%) Polymorphic Lymphoproliferative Disorders (PLD) and 57/76 (75%) Malignant Lymphoma (ML). Fifty-nine of seventy-eight (75%) patients developed late onset PTLD (> 12 months from transplant). Among patients tested for EBER, 49/72 (68%) were EBV positive. While EBER positive PTLDs were heterogeneous with regard to time of occurrence and histological characteristics (6 PH, 11 PLD, 31 ML, 1 not classified), all EBER negative PTLDs were late onset ML. Diagnosis was obtained post-mortem in 9/78 patients. The treatment was tailored according to clinico-pathological features: 52/69 (75%) patients received a single agent or a combined regimen of chemotherapy, associated with antiviral drugs in EBER positive forms. Rituximab has been introduced in the therapeutic schedule of CD20+ PTLDs since 2000. It was administered to 24 patients, combined with chemotherapy in all but 2 cases.

Radiation and surgery were used when indicated. Eleven patients died early, before any treatment was completed (median time 18 days, range 5-56); 2/69 patients were lost at follow-up. Therefore, a total of 56 patients underwent their scheduled treatment and were evaluable for the outcome. We observed 10/56 deaths because of treatment related toxicity or disease progression. Complete remission (CR) was achieved in 45/56 (80%) patients. Of these, 8 (18%) relapsed, mostly responsive to second line therapy (7/8 patients), and 10 patients died because of late treatment-related toxicity or infection. One patient is still alive in partial remission (PR) at 23 months. The median survival time of evaluable patients was not reached at 3250 days (see Figure 1). *Conclusions*. We can confirm that PTLD is a significant cause of mortality in solid organ transplant recipients: in our study the overall mortality rate was 45% (31/69) and the exitus mostly represents an early event, occurring within 6 months from diagnosis in 20/31 patients (64%). Nevertheless, timely and tailored treatment of the disease and its complications warrants long-lasting complete response with low relapse rate. PTLDs are characterized by wide clinico-pathological variability and represent a heterogeneous disease: better knowledge of biological parameters (e.g. EBV pathogenic role, donor or recipient PTLD origin, immunologic status of patients) could help to stratify our patients in different risk groups and could allow more appropriate treatment.



0213

RISK OF SECOND CANCER IN NON-GASTRIC MARGINAL ZONE B-CELL LYMPHOMA OF MALT: A POPULATION-BASED STUDY FROM NORTHERN ITALY

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Background. Marginal zone B-cell lymphomas (MZL) of MALT show a peculiar relationship with the triad autoimmunity-infection-immunosuppression. For this reason, these lymphomas have been studied for the risk of second cancers. Most series reported so far regard patients with gastric MALToma, while data on nongastric MZL of MALT are lacking. *Aim.* To define the risk of second cancer in nongastric MZL of MALT in a population-based study from Northern Italy. *Methods.* We studied the prevalence of second cancers in a series of 157 patients with nongastric MZL of MALT consecutively diagnosed in two haematological Institutions of the Northern Italy region Lombardia. We compared the occurrence of second cancer with respect to the general population by calculating the standardized incidence ratio (SIR), with the age- and sex-specific incidence rates of the Cancer Registry of Lombardia as a reference. *Results.* A history of 30 additional neoplasms was documented in 29 patients (18%) (18 females and 11 males): 21 previous, 3 concurrent, and 6 subsequent. The malignancies were: 25 solid tumors, 2 hematological diseases (1 Hodgkin's lymphoma and 1 essential thrombocythemia), 3 non-melanoma *in situ* skin cancers. One patient had two malignancies (breast cancer and essential thrombocythemia), both prior to the diagnosis of cutaneous lymphoma. The sites of solid cancers were: 8 breast, 4 endometrium, 4 skin, 3 thyroid, 2 lung, 1 prostate, 1 colon, 1 small intestine, 1 salivary gland, 1 bladder, 1 ovary and 1 stomach. In 4 patients the site of cancer and lymphoma was the same. For the entire group, the SIR of an additional malignancy was 0.8 (95% CI: 0.55-1.17, p=0.2). The relative rate of an additional malignancy was 0.7 for males (95% CI: 0.39-1.26, p=0.2) and 0.89 for females (95% CI: 0.55-1.46, p=0.6). The comparison of risks between males and females was not significant (SIR ratio 1.28, 95% CI: 0.59-2.76, p=0.5). After excluding non-melanoma skin cancers, the SIR of a second tumor was 0.75 (95% CI: 0.5-1.12, p=0.2). The relative rate of a second tumor was 0.6 for males (95% CI: 0.31-1.15, p=0.1) and 0.89 for females (95% CI: 0.54-1.47, p=0.6). The comparison of risks between males and females was not significant (SIR ratio 1.49, 95% CI: 0.65-3.4, p=0.3). After excluding all previous malignancies, the SIR of a second cancer was 1.32 (95% CI: 0.69-2.55, p=0.4). All concomitant and subsequent malignancies were invasive tumors. The relative rate of a second cancer was 1.46 for males (95% CI: 0.61-3.51, p=0.4) and 1.19 for females (95% CI: 0.44-3.16, p=0.7). The comparison of risks between males and females was not significant (SIR ratio 0.81, 95% CI: 0.22-3.02, p=0.8). *Conclusions.* These data demonstrate that patients with nongastric MZL of MALT are not at increased risk for second cancer compared to the general population of the same geographical area. However, since nongastric MALT lymphoma is a long-lasting disease of advanced age with high risk of relapse, a careful clinical follow up is always warranted.

0214

ACHIEVEMENT OF MOLECULAR REMISSION AFTER FIRST LINE TREATMENT PROLONGS SURVIVAL IN FOLLICULAR LYMPHOMA PATIENTS

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Backround. However is follicular lymphoma (FL) still considered conventionally incurable disease, prolonged complete remissions were reported. Results of recent studies suggest, that patients who achieve complete remission (CR) with PCR bcl-2/IgH negativity (molecular remission, CRm) have better long term outcome. *Aims.* To evaluate whether achieving of molecular remission after first line treatment have an impact on disease free (DFS) and overall (OS) survival in all risk subgroups, previously untreated, follicular lymphoma patients. *Methods.* 104 (pts) with FL were diagnosed and treated in our department during last 8 years. All of them were examined with a qualitative PCR (bcl-2/IgH) from bone marrow (BM). Bcl-2/IgH (MBR, mcr or long-distance PCR) positivity was observed in 55 pts (57%) at the time of diagnosis. 91% of bcl-2/IgH+ pts had an advanced disease stage (III/IV), BM involvement was present in 43.5% bcl-2/IgH+ pts. First line treatment was stratified acording generally used risk factors (FLIPI, GELF, β-2-m level, bulk disease). Patients under 60 (65) y.o. with high risk disease (FLIPI ≥3 or additional risk factors) were indicated to stem cell transplantation (SCT). 19 patients underwent autologous and 1 patient allogenic SCT. 36 patients were treated conventionally (CHOP or fludarabine based regimens). Rituximab was administred as first line concomitant chemo-immunotherapy in 21 pts (equally in both groups). PCR (BM and/or peripheral blood) was reevaluated on the day +100 after SCT or at the point of restaging and during follow-up every 6 months.

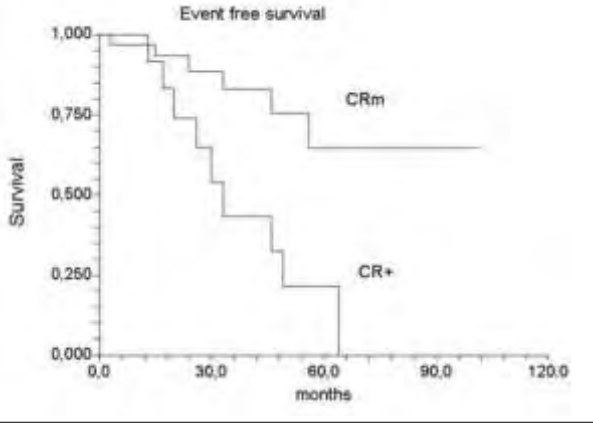

Figure 1. Event free survival: impact of residual disease.

Results. After first line treatment 35/55 (64%) pts achieved CRm, 13 pts (24%) CR+, 9 (16%) PR+ (partial response). After SCT (81.8%) pts attained CRm (100% receiving rituximab in the 1st line). Median follow up is 50 months (mo). 23/55 (41.8%) pts relapsed or progressed (median PFS 30 mo), 11 pts died - one in CRm due to acute graft versus host disease, the other due to progression of the disease. At present 32/35 CRm pts are still in CRm, whereas 6/11 pts in CR+ relapsed. Median disease free survival (DFS) was longer in CRm pts than in CR+ pts (median EFS 33 mo vs not reached, p 0.0024). Median DFS in patients with or without autologous stem cell transplantation was not significantly different. Patients in CRm have longer overall survival (64 mo vs not reached, p 0.05) compared to pts in CR+ and PR. *Summary.* Molecular remission after first line treatment have an impact on disease free and overall survival in all risk subgroups of FL patients. Persistent PCR bcl-2/IgH positivity is asociated with high risk of relapse and additonal (maintenance) treatment should be considered.

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0215

CLINICO-BIOLOGICAL CHARACTERISTICS OF PATIENTS WITH DIFFUSE LARGE B CELL LYMPHOMA AND HEPATITIS C VIRUS INFECTION

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Background. The infection with Hepatitis C Virus (HCV) is involved in the etiology of some subtypes of non Hodgkin's lymphoma (NHL) as diffuse large B cell lymphoma (DLBCL). *Aims.* We tried to analyze the clinico-biological characteristics of a group of 82 patients with DLBCL associated with HCV infection. *Methods.* We did a retrospective analysis of the clinical and biological profile of 82 patients hospitalized in the Hematology Clinic during 1993-2003. All patients were HIV negative and they were positive at diagnosis for the HCV antibodies Elisa method. The statistical analysis was performed with the special programs EPI INFO 6 and INSTAT. *Results.* The characteristics of the diffuse large cell lymphoma group that we studied were: medium age - 52,2 years; males/females=1/1; extranodal determinations - 48 cases (58.5%)- primitive extranodal 25 cases (30.5%) and secondary extranodal 23 cases (28%); B signs - 66 patients (80,5%); IK < 70 - 40 cases (48,8%); IPI at diagnosis was 16% low, 24% int.low, 35% int/high and 25% high; Bulky disease - 35 cases (42,7%); clinical stage I, II/III, IV=40/41; medullar determination - 14 cases (17,2%); LDH = 672,024±598,128 U/l; ESR = 50,45±37,73 mm/h; Ki67 = 51,90±22,16. The transformation from a low grade lymphoma in a DLBCL was 10% and primary mediastinal DLBCLwas 5%. The most important extranodal determinations were the stomach, the spleen, the liver, the skin. The treatment was CHOP or CHOP-like regiments. A small number of cases received CHOP and Rituximab. 11% of patients with severe liver dysfunction received monochemotherapy or radiotherapy only. In 5% of DLBCL and HCV positive patients the chemotherapy was discontinuous because of the hepatic failure. Medium follow-up was 48 month for the survivors and the overall survival at 5 years was 59%, while failure free survival at 5 years was 34%. *Conclusions.* It is important to recognize the clinical and biological features of DLBCL with HCV positive patients for bigger groups, which could clarify the connection between HCV infection and aggressive non Hodgkin lymphomas. It is also necessary to continue the study in order to evaluate the differentiate survival of nodal and extranodal types of lymphomas.

0216

RITUXIMAB IN INDUCTION TREATMENT AND IN HIGH DOSE CHEMOTHERAPY PRIOR TO AUTOLOGOUS STEM CELL TRANSPLANTATION AS FIRST LINE THERAPY IN STAGE III-IV DIFFUSE LARGE B-CELL LYMPHOMA AT POOR PROGNOSIS

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Background. We investigated efficacy and safety of adding Rituximab (R) to induction and intensified HDC as part of first line treatment in pts with aa-IPI at Intermediate-High (IH) or High (H) risk with B-DLCL at diagnosis, comparing two groups of pts enrolled in two non-randomized phase II clinical trials with up-front HDC and ASCT with or without R. Aims and *Methods.* 118 previously untreated pts <61 years with B-DLCL, stage III-IV at aa-IPI IH or H risk were treated: 41 pts were enrolled into HDC trial (control group; August 1991-August 1995) and 77 pts into R-HDC trial (study group; January 2001-December 2004). Treatment in R-HDC study group consisted in an induction treatment lasting two months with four courses of R-MegaCEOP chemotherapy (R 375 mg/m^2 day1, CTX 1200 mg/m^2 + EPI 110 mg/m^2 + VCR 1.4 mg/m^2 day3 and PDN 40 mg/m^2 days3-7) every 14 days with G-CSF support; then two courses of intensified chemoimmunotherapy R-MAD (Mitoxantrone 8 mg/m^2 + ARAC 2000 mg/m^2 /12h + Dexamethasone 4 mg/m^2 /12h for 3 days and R 375 mg/m^2 day4 and before PBSC harvest) followed by ASCT with BEAM as conditioning regimen. Treatment in HDC control group was an induction treatment lasting two months with MACOPB x 8 weekly infusions followed by the same intensified and HDC regimens (MAD x 2 courses + BEAM and ASCT). IF RT was given to areas of previous bulky disease in both trials. *Results.* Pts characteristics in both trials were comparable with no statistically significant differences: median age was 45 years (19-60); 51% were at H risk; 36% had bone marrow (BM) involvement, 80% LDH>normal and 42% extranodal sites>1. Complete Response at the end of the treatment was: 60 pts (78%) in R-HDC group and 28 (68%) in HDC group (p=.25). Failures (17% vs 25%) and toxic deaths (5% vs 7%) were comparable between the two groups (R-HDC vs HDC). Short-term toxicity appeared similar. NO MDS or ANLL or solid tumours were reported in both arms. No differences were observed in neutrophils >500/mm^3 and platelets >50000/mm^3 engraftment; median times in R-HDC vs HDC were: 9 vs 10 and 15 vs 16 days. Median follow-up was 36 months in study group and 72 months in control group. Three-year failure-free survival (FFS) and 3-yr overall survival (OS) rates in R-HDC group vs HDC group were: FFS 64% vs 46% (p=.016); OS 80% vs 54% (p=.004). A better outcome for pts treated with R-HDC was confirmed in both IPI groups (IH and H risk). A Cox's model was performed to adjust the effect of treatment for competing risk factors (age, IPI, BM involvement, number of extranodal sites). In this multivariate analysis the risk of failure and death was confirmed as significantly reduced in R-HDC group: adjusted hazard ratio (R-HDC vs HDC) was 0.56 (95% CI=0.30-1.01, p=.05) for FFS and 0.42 (95% CI=0.21-0.88, p=.02) for OS. *Conclusions.* these results suggest that the addition of Rituximab to induction and intensified chemotherapy before BEAM and ASCT is effective and safe in B-DLCL at poor prognosis.

0217

EFFECTS OF PRE-TRANSPLANTATION TREATMENT WITH RITUXIMAB ON OUTCOMES OF AUTOLOGOUS STEM-CELL TRANSPLANTATION FOR DIFFUSE LARGE B-CELL LYMPHOMA

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Background. Rituximab (R) in combination with chemotherapy (CHT) has become standard treatment for patients (pts) with diffuse large B-cell lymphoma (DLBCL). However, there are limited data concerning the comparison of the use of R-CHT and CHT alone before high-dose therapy (HDT) either as a part of induction regimen or as a part of salvage treatment. *Aims.* We retrospectively analysed the efficacy of R-CHT versus CHT without R followed by HDT and autologous stem cell transplantation (ASCT) in patients with DLBCL. *Methods.* Out of 127 pts with DLBCL who underwent HDT with ASCT, 59 pts received R as part of chemotherapy regimen (R-CHT group): 32/59 (54%) received HDT in 1st CR. 68 pts received CHT without R (CHT group): 14/68 (21%) received HDT in 1st CR (p<0.0001). Patient characteristics were comparable in both groups with the exception of status at HDT, indi-

cation to HDT (induction vs salvage therapy) and type of salvage regimen. Higher proportion of patients received HDT as a part of induction therapy in R-CHT 69,5% vs 48,5% in CHT group (p=0.01). The majority of pts in R-CHT group received salvage regimen ICE. Regimens ESHAP and IVE were mostly used as a salvage therapy in CHT group. Median follow-up is 2.2 y (range 0.5-4.0) in R-CHT group and 7.3 y (range 2.1-11.0) in CHT group. *Results.* At 2 years from the date of transplantation, the estimated overall survival (OS) was 81% in R-CHT group vs 60% in CHT group (p=0.03) and the event-free survival (EFS) was 75% vs 56% (p=0.02). The results remain significant while analyzing data for pts transplanted in 1st CR in R-CHT vs CHT group, OS 87% vs 57% (p=0.04), EFS 84% vs 57% (p=0.04) at 2 years. The differences were however not significant for pts who underwent HDT for relapse in R-CHT vs CHT group: OS 69% vs 55% and EFS 53% vs 48% at 2 years. *Conclusion.* Our analysis suggests that rituximab plays a significant role in pretransplant therapy in pts with poor risk factors treated with HDT in 1st CR (EFS, p=0.04, OS, p=0.04). Rituximab seems to improve the outcome of CR pts, it might be important how is the CR reached (with or without antibody). The difference is not however significant for pts treated with HDT in relapse or progression. The role of rituximab in this subset of pts could be in the improvement of salvage therapy results in order to increase the number of pts who are able to undergo HDT and ASCT.

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0218

COMPARATIVE ANALYSIS OF TREATMENT OUTCOMES WITH CHOP REGIMEN, ETOPOSIDE PLUS CORTICOSTEROID AND PREDNISOLONE IN ADULT PATIENTS WITH HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS: BASED ON UNDERLYING DISEASES

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Background. The outcome of CHOP treatment in the case of lymphoma-associated hemophagocytic lymphohistiocytosis (LAHLH) and EBV-associated HLH (EBV-HLH) has rarely been reported. *Aims.* The present study analyzed the treatment outcomes for CHOP chemotherapy as well as etoposide combined with corticosteroid (Eto-CS) and prednisolone (PRS) in adult patients with EBV-HLH and LAHLH. *Methods.* 46 adult patients older than 16 years of age were diagnosed with HLH. Among these patients, 30 treated with CHOP chemotherapy (n=18), Eto-CS (n=6), and PRS (n=6) were reviewed retrospectively. *Results.* With CHOP chemotherapy, complete remission (CR) was achieved in 5/18 patients (27.8%), partial remission (PR) in 5/18 (27.8%), and the overall response rate was 55.6%. With Eto-CS therapy, PR was achieved in 3/6 patients (50%), however no CR was achieved. With PRS therapy, CR was achieved in 1/6 patients (16.7%) and PR in 1/6 (16.7%). The median response duration (RD) was not reached and the 3-year estimated RD was 68.57% for the CHOP chemotherapy, while the median RD was three weeks for the Eto-CS therapy and one week for the PRS therapy, with a median follow-up of 132 weeks. The median duration for the overall survival (OS) was 16 weeks and the 3-year estimated OS rate 40.63% for the patients treated with CHOP therapy, yet only four and two weeks for the patients treated with Eto-CS and PRS, respectively (p=0.0016). *Conclusions.* CHOP chemotherapy seemed to be useful in adult patients with LAHLH and EBV-HLH. Additional treatment including stem cell transplantation may also be needed, especially for patients with poor prognostic factors.

Myeloma and other monoclonal gammopathies I

0219

PP2500 MRNA, A SPLICE VARIANT OF THE MULTIPLE ANKIRIN REPEAT SINGLE KH DOMAIN (MASK), IS HIGHLY EXPRESSED IN PLASMA CELLS OF MULTIPLE MYELOMA

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Background. The Ankyrin (ANK)-repeat is one of the most common protein sequence motifs, which leads itself to variation in overall domain size by simple sequence duplication or deletion. The Mask (Multiple Ankyrin Repeats Single KH domain) gene, which codifies an ANK-repeat protein, is located in chromosome 5(q31.3) and it is composed of 39 exons . It generates isoforms by alternative 3'splicing. The first splice variant (hMask) lacks the 10A exon of the Mask gene, generating a mRNA containing 34 exons. The other, Mask-BP3ARF, results from fusion of splice variant hMask, with the two last exons of the gene Eif4Ebp3 (exons B and C) and an intermediate exon (exon 0), generating 36 exons. Recently, a new splice variant, denominated PP2500, was deposited in the data base GeneBank, it presents the first 10 exons, homologous to Mask mRNA with a poly(A+) signal and it is a new splice variant of Mask. In Drosophila, MASK protein seems to interact with members of the Receptor Tyrosine Kinase (RTK) signalling pathway and loss of this interaction increases programmed cell death, reduces cell proliferation, inhibits photoreceptor differentiation, affects RTK dependents processes but does not affect MAPK (Mitogen Activated Protein Kinase) activation. However, the biological functions of these proteins in humans remain still unknown. Aim:The aim of this study was to investigate the expression of Mask splice variants in multiple myeloma (MM).*Methods.* Fifteen patients with MM and 3 normal donors participated in this study. Total RNA was extracted from positively selected plasma cells in magnetic column, by Macs Microbeads antibody anti-CD138 and the percentage of purity of plasma cells varied from 78.38% to 96.02% (average 87.95%). We used as control, total RNA from positively selected plasma cells, from a culture of B normal lymphocytes of bone marrow donors (purity 88.69%). The complementary DNA (cDNA) was analyzed by Real-time detection of amplification, performed in an ABI 5700 Sequence Detector System using SybrGreen PCR Master Mix (qPCR). The b-actin gene was used as endogenous control of the reaction. *Results.*The mean expression of the mRNA of the hMask and Mask-BP3ARF genes were 3 and 4 times increased, respectively, compared with control. The mean expression of the PP2500 mRNA was 14 times increased, compared with control. Quantification of hMask, Mask-BP ARF and PP2500 mRNA was not influenced by age, gender, ethnic origin, stage of the disease, B2-microglobulin, serum creatinine and lactate dehydrogenase values (Fisher´s exact test, p>0.05). Previously we demonstrated that MASK is associated with SHP2, a protein tyrosine-phosphatase. *Conclusions.*In MM, SHP2 mediates the anti-apoptotic effect of Interleukin-6 (Chauhan *et al.* JBC 275: 27845, 2000). Interleukin-6 triggers proliferation of MM cells via the MAPK cascade, which includes SHP2 activation. Thus, the increased expression of Mask splice variants in plasma cells of MM suggests that their proteins may be involved in this signaling pathway and provide an insight for novel treatment approaches in MM.

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0220

A PHASE II STUDY OF THALIDOMIDE, DEXAMETHASONE AND PEGYLATED LYPOSOMAL DOXORUBICIN (THADD) FOR UNTREATED PATIENTS WITH MULTIPLE MYELOMA AGED OVER 65 YEARS

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Background. No standard therapy have been yet identified for elderly patients with multiple myeloma (MM) despite two third of cases affected by this incurable malignancy are older than 65 years. The combination melphalan-prednisone yelds unsatisfactory results and high-dose therapy, despite feasible also in elderly patients, can be an unavailable option because of pre-existing medical comorbidities. Improvements in the outcome of elderly MM patients have been obtained using thalidomide as single agent or in combination with dexamethasone or conven-

tional chemotherapy. *Aims.* We report the results of a phase II study including 50 newly diagnosed patients with symptomatic MM older than 65 years regardless of comorbidities, performance status and renal function. *Methods.* All patients received thalidomide 100 mg/day continously, pegylated liposomal doxorubicin 40 mg/m^2 on day 1 every 28 days, dexamethasone 40 mg on days 1-4 and 9-12 (ThaDD). They also were given warfarin 1.25 mg/day as antithrombotic prophylaxis and ciprofloxacin 250 mg twice daily after a high incidence of infections was recognized. Median age was 71.5 years (range 65-78) and 64% were older than 70 years. Thirty-nine patients (78%) had clinical stage III, 37 (74%) ISS[3] 2 and 7 patients (14%) a serum creatinine level > 2 mg/dL. Moreover, unfavourable cytogenetics were detected in 33% of patients with a valuable test. *Results.* According to EBMT criteria, 17 (34%) patients achieved CR, 7 (14%) nCR, 5 (10%) VGPR, 15 (30%) PR and 5 (10%) MR resulting an ORR of 98%. Seven patients (14%) underwent autologous stem cell transplantation. Median PFS, EFS and OS were not reached whereas PFS, EFS and OS projected at 3 years were 60%, 57% and 74%, respectively. Patients achieving [3] VGPR had a significantly better PFS, EFS and OS than patients who did not. The compliance to treatment was high and only two patients refused to continue therapy because of the occurrence of pulmonary embolism and septic shock. Grade 3-4 neutropenia occurred in 6 (12%) patients. Infectious complications occurred in 25% of patients before antibiotic prophylaxis was given whereas they developed in 7% after ciprofloxacin has been added to the protocol. Grade 3-4 nonhematological side effects were mainly attributable to thalidomide and consisted of constipation (4%), fatigue (6%) and tremors (4%). Regarding toxicity due to pegylated liposomal doxorubicin, 2 patients experienced grade 3-4 mucositis and one grade 3 palmar-plantar erythrodysesthesia. Venous thromboembolic events occurred in 7 patients (14%) but only one patient experienced pulmonary embolism. *Conclusions.* Our study demonstrates that the combination low-dose thalidomide, pegylated liposomal doxorubicin and high-dose dexamethasone is very effective in the treatment of elderly patients with MM since it induces an ORR and particularly a CR rate higher than those reported with all other thalidomide-based regimens. It results well tolerated also by oldest fragile patients and thrombotic as well as infectious complications can be prevented by adequate prophylaxis.

0221

MONOKINE-INDUCED BY INTERFERON-γ SERUM LEVELS ARE A MARKER OF DISEASE LOAD AND CORRELATE WITH PROGNOSIS IN MULTIPLE MYELOMA

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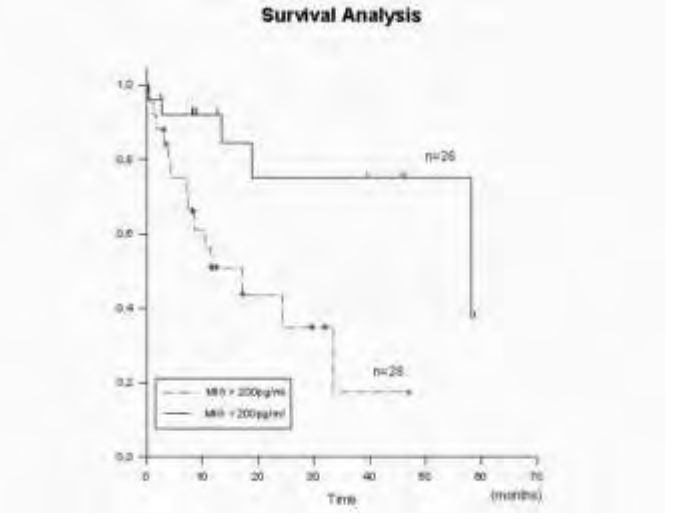
Background. Monokine-induced by interferon-γ (MIG) is a chemokine known to be produced by monocytes and macrophages in response to interferon-γ, and acts as a chemoattractant to T-lymphocytes and other inflammatory cells. Besides its role in the host immune response to infections and neoplastic disease, MIG has also been implicated as chemokine acting in an autocrine loop to stimulate tumor cells through its receptor CXCR3. Myeloma cells are known to express CXCR3 (Pellegrino *et al.,* 2004), however it is unclear if MIG is of biological significance in myeloma *in vivo*. *Aims.* We have shown recently that multiple myeloma oncogene 1 (MUM1) expression in myeloma cells correlates with prognosis in this disease (Heintel *et al.*, 2005), and MUM1 is known to upregulate MIG gene expression in B cell malignancies (Uranishi *et al.*, 2005). This led us to evaluate the potential prognostic significance of MIG serum levels in a series of myeloma patients. *Methods.* MIG serum levels were determined by a commercially available ELISA (R&D Systems) in a series of 54 newly diagnosed myeloma patients. Serum from 8 healthy volunteers, 4 patients with osteoporosis, and 4 patients with chronic obstructive pulmonary disease (COPD) were used as controls. *Results.* Median MIG serum level was 38.3 pg/ml in healthy volunteers, range 22.7-52.79 pg/mL. In patients with osteoporosis and COPD the levels were higher (median 133.0, range 47.2-202.3 pg/mL; and median 84.9, range 25.32-200.2 pg/mL, respectively). In 54 newly diagnosed myeloma patients the median MIG level was 219.8, range 27.6-1966.0 pg/mL. When myeloma patients were stratified according to a MIG level < 200 and MIG > 200, a highly significant survival difference for the 2 cohorts was observed. While median survival was 58.2 months for patients with MIG < 200, patients with high MIG serum levels (> 200) had a survival of only 17.0 months (p=0.00409; see Figure). Serum-MIG levels correlated with markers of disease burden, including β2-microglobulin levels and extent of bone marrow plasma cell infiltration. MIG showed a negative correlation with hemoglobin and albumin levels. Interestingly, no correlation was found with C-reactive protein levels, indicating that MIG is not associated with an inflammatory response in myeloma. Preliminary experiments show that MIG mRNA is expressed in 1 out of 4 myeloma cell lines, with upregulation of expression seen after stimulation with interferon-γ in the positive line, but not in those without baseline MIG expression. *Summary/Conclusions.* MIG serum levels correlate with markers of disease burden in myeloma and high MIG levels are associated with a poor outcome in this disease.



0222

VAD-DOXIL VS.VAD-DOXIL PLUS THALIDOMIDE AS INITIAL TREATMENT IN MYELOMA PATIENTS: INTERIM ANALYSIS OF A MULTICENTER RANDOMIZED TRIAL OF THE GREEK MYELOMA STUDY GROUP

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Background. VAD-doxil and VAD-doxil plus thalidomide have already been separately evaluated, as initial cytoreductive treatment in multiple myeloma, in two previous clinical trials of our study group. Both regimens proved effective yielding overall (complete and partial) response rates of 61.3% and 74% respectively, while toxicity remained acceptable in both studies. *Aims.* To compare the efficacy and toxicity of these two regimens in the context of a multicenter randomized clinical trial. Results of an interim analysis are presently reported. *Methods.* Patients randomized in arm A received vincristine 2 mg IV, liposomal doxorubicin 40 mg/m^2 IV in a single dose on day 1, and dexamethasone 40 mg PO daily for 4 days. The regimen was repeated every 4 weeks. Dexamethasone was also administered on days 15-18 of the first cycle. Patients randomized in arm B received additionally thalidomide 200 mg PO daily at bedtime. Response to treatment was the primary objective of the study and was evaluated after the completion of 4 cycles. Subsequently, patients were allowed to proceed to high dose chemotherapy or to receive two additional cycles of the same regimen. Response and toxicity were evaluated according to EBMT and NCI criteria respectively. Patients' characteristics, response and toxicity rates were compared using two-independent- samples tests and x2 tests. *Results.* Between June 2002 and December 2005, 230 patients entered the study, 115 randomized in each arm. To date, 198 patients are evaluable for toxicity and 160, 80 in each arm, for efficacy. The two treatment groups were well-balanced regarding the usual prognostic characteristics. On an intention- to- treat basis, overall response rate was 66.3% and 81.3% in arms A and B respectively (p=0.048). Neutropenia, thrombocytopenia, infections, mucositis, palmar-plantar erythrodysesthesia, deep venous thrombosis and early mortality were not significantly different (p>0.05) between arms A and B

(13% vs. 15%, 8.5% vs. 10%, 7.5% vs. 5%, 5% vs. 4%, 6.3% vs. 5%, 3.8% vs. 9.5% and 6.3% vs. 5% respectively). Constipation, peripheral neuropathy, dizziness/somnolence, skin rash and edema were significantly higher (p<0.05) in arm B compared to arm A (57% vs. 10%, 46% vs. 13.8%, 54% vs. 0%, 13% vs. 0%, 10% vs. 2% respectively). *Conclusions.* VAD-doxil plus thalidomide compared to VAD-doxil alone, yields higher response rates in previously untreated myeloma patients. Nevertheless, the increased toxicity associated with the addition of thalidomide to VAD-doxil, should be counterbalanced against the increased response rate.

0223

THE COMBINATION OF BORTEZOMIB, MELPHALAN, DEXAMETHASONE AND INTERMITTENT THALIDOMIDE (VMDT) IS AN EFFECTIVE REGIMEN FOR RELAPSED/REFRACTORY MYELOMA AND REDUCES SERUM LEVELS OF RANKL, MIP-1 A AND ANGIOGENIC CYTOKINES

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Background. Interactions between myeloma (MM) cells and marrow microenvironment are crucial for myeloma growth and resistance to anti-myeloma therapy. Bortezomib (VELCADE®; V) and thalidomide (T) have proven anti-MM effect and exert their action partly through perturbation of the MM microenvironment. Furthermore, bortezomib enhances the cytotoxic potential of other agents, such as melphalan (M), and dexamethasone (D) in resistant cell lines. *Aims.* We hypothesize that combining VT (to target both MM cells and microenvironment) with M/D may help overcome resistance and increase clinical efficacy of these agents in relapsed/refractory disease. The aim of this phase II study was to determine the efficacy and safety of the VMDT regimen and its effect on angiogenesis and bone remodeling in relapsed/refractory MM. *Methods.* Bortezomib (1.0 mg/m^2) was given iv, on days 1, 4, 8, and 11 of a 28-day cycle; oral melphalan (0.15 mg/kg) was administered on days 1-4, while thalidomide (100 mg/day) and dexamethasone (12 mg/m^2) were given on days 1-4 and 17-20 every 4 weeks, for 4 cycles. Responders and patients with SD continued for up to 8 cycles. Effect of VMDT on angiogenesis was evaluated by measuring the serum levels of angiogenic cytokines, such as VEGF, angiogenin, angiopoietin-2, and basic fibroblast growth factor (bFGF) at baseline and after 4th and 8th cycle. Bone remodeling was studied by the measurement of a series of serum indices: i) osteoclast stimulators [sRANKL, osteoprotegerin (OPG), osteopontin, macrophage inflammatory protein-1 α (MIP-1α)], ii) bone resorption markers (CTX, TRACP-5b), and iii) bone formation markers [bone-specific alkaline phosphatase (bALP), osteocalcin (OC), CICP]. Forty-four pre-treated patients have been enrolled in this ongoing study including 25 patients treated during refractory relapse. Median time from 1st treatment to VMDT was 38 months. The median number of previous treatment was 2 (range: 1-6), including melphalan (47% of patients), thalidomide (56%), dexamethasone (100%), bortezomib (9%) and ASCT (32%). *Results.* Among 41 patients evaluable for response so far, 27 (65%) achieved an objective response (CR 9% and PR 56%). Furthermore, 5 patients (12%) achieved a MR and 5 SD. Median time to response was 36 days. Adverse events included fatigue (52%), thrombocytopenia (20% grade 3/4), neutropenia (8% grade 3/4), anemia (7% grade 3), neuropathy (47% grade 1/2, and 6% grade 3), infections (47%, including 4 HZV cases), and hyponatremia (18%). No patient experienced DVT, while 2 patients died due to sepsis and one due to necrotizing fasciitis. At baseline, MM patients had increased serum levels of sRANKL, sRANKL/OPG ratio, MIP-1α, CTX, VEGF, angiogenin, angiopoietin-2, and bFGF (p<0.01) compared with controls (21 healthy, age- and gender-matched, individuals), while serum levels of bALP, and OC were reduced (p<0.0001). Our preliminary analysis has shown that sRANKL, sRANKL/OPG ratio, MIP-1α, CTX, and all angiogenic cytokines' levels reduced after 4 (p<0.001) and 8 cycles of treatment (p<0.01) in all patients. Responders tended to have a higher reduction of both serum sRANKL and MIP-1α compared with non-responders. *Conclusions.* VMDT provided encouraging evidence of antitumor activity in relapsed/refractory MM, with manageable toxicities and alterations in cytokines conducting interactions between myeloma and stromal cells.

0224

BORTEZOMIB DEMONSTRATES SUPERIOR SURVIVAL COMPARED WITH HIGH-DOSE DEXAMETHASONE AND HIGHER RESPONSE RATES AFTER EXTENDED FOLLOW-UP IN THE APEX TRIAL IN RELAPSED MULTIPLE MYELOMA

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Background. In the open-label, international, multicenter phase 3 APEX trial, 669 patients with relapsed multiple myeloma (MM) following 1-3 prior therapies were randomized to receive bortezomib (VELCADE) or dexamethasone (Dex). Patients with progressive disease on Dex were eligible to cross over to bortezomib. Patients receiving bortezomib achieved significant improvement in survival, time to progression (TTP), and response rate (CR + PR, EBMT criteria). Consequently, the Dex arm was halted early and all patients receiving Dex were allowed to cross over to bortezomib. *Aims.* To update survival data for both the bortezomib and Dex arms of the APEX trial, and to update efficacy data for the bortezomib arm. *Methods.* Updated overall and 1-year survival rates were analyzed for both arms based on median follow-up of 22 months in surviving patients and deaths in 44% of patients. Updated response rate, time to response (TTR), duration of response (DOR), and TTP were analyzed for the bortezomib arm with extended follow-up of approximately 14 months compared with the initial analysis. Timing of best response to bortezomib was analyzed in terms of EBMT criteria and M-protein reduction, and DOR was analyzed according to best M-protein reduction. Matched-pairs analyses were performed to compare survival and response rate in patients receiving bortezomib earlier (bortezomib arm) or later (Dex arm patients who crossed over to bortezomib arm). *Results.* Patients received a median of 6 cycles of bortezomib. Median survival was 29.8 months vs 23.7 months (p=0.0272), and 1-year survival rate was 80% vs 67% (p=0.0002), for bortezomib vs Dex, despite >62% of Dex patients crossing over to bortezomib. With extended follow-up, overall response rate by EBMT criteria with bortezomib improved from 38% in the initial analysis to 43%, and CR improved from 6% to 9%, with 56% of responders experiencing an improved response after cycle 2, and 54% of responders achieving first response after cycle 2. The proportion of patients achieving maximum M-protein reduction continues to increase over the entire course of study-specified treatment (up to 8 cycles). Median TTP (6.2 months), TTR (1.4 months), and DOR (7.8 months) with bortezomib were unchanged compared with initial analysis. Median DOR was 11.5 months in patients with 100% M-protein reduction, and 7.6 months in patients with ≥50% but <100% M-protein reduction. Overall response rate and median survival in patients receiving bortezomib earlier vs later were: 44% vs 34%, and not reached vs 16.4 months, respectively (Table). *Conclusions.* After extended follow-up, significantly longer survival with bortezomib compared with Dex was confirmed, despite substantial crossover from Dex to bortezomib. Response rates are higher, with many patients achieving best responses after longer duration of therapy. Patients achieving 100% M-protein reduction tended to have a longer DOR. Additionally, patients receiving bortezomib earlier appear to have a higher response rate and longer survival.

	Bortezomib earlier (n=102)	*Bortezomib later (n=102)*
Response rate,%	44	34
CR	9	7
PR	35	27
near CR	6	10
Median survival, momths	Not reached*	16.4*

**Hazard ratio = 0.75; p= 0.1722*

0225

SIMPLE, BUT SIGNIFICANT FACTORS FOR SURVIVAL AFTER AUTOLOGOUS TRANSPLANTATION IN 181 MULTIPLE MYELOMA PATIENTS: A SINGLE CENTRE EXPERIENCE

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Background. Autologous stem cell transplantation (ASCT) has an established role in the treatment of symptomatic multiple myeloma (MM). Reliable and simple staging of MM is important for accurate prognostic evaluation and for the comparison of data from different clinical trials. Attempts to improve the widely accepted Durie-Salmon (DS) staging system have led to the development of numerous new prognostic systems, that have not been universally accepted. Recently new International Staging System (ISS) was presented. It has shown promise in patients (pts) treated by conventional as well as high-dose chemotherapy and is based on a simple combination of serum β2microglobulin (B2M) and albumin (alb) values (stage 1 = B2M under 3.5 mg/L and alb above 3.5 g/dL; stage 2 = B2M under 3.5 mg/L and alb under 3.5 g/dL, or B2M from 3.5 mg/L to 5.5 mg/L; stage 3 = B2M above 5.5 mg/L). *Aims.* The aim of our analysis was to evaluate the impact of selected clinically significant parameters for the survival of MM pts after ASCT including both ISS and DS systems in our set of pts. *Methods.* We have retrospectively evaluated 181 pts with MM undergoing autologous transplant (ASCT) in our centre between 1995-2004, median follow up from ASCT is 59 months, range 8-107 months. All pts had the same pretransplant therapy and were transplanted to one year after diagnosis. *Results.* Following ASCT, 52 pts (29%) were in complete remission (CR) and 113 pts (62%) in partial remission (PR). The median progression-free (PFS) and overall (OS) survival from transplant were 26.7 and 72.6 months, respectively. Seventeen pts (9%) are in CR and disease free over 5 years after ASCT (median follow up of this subgroup is 87 months, range 63-112). Differences in survival among pts with clinical stages according to DS system were not statistically significant (p=0.214). Patients with clinical stages according to ISS were significant differences in survival (p under 0.001): stage I (71 pts) ' median was not yet reached, stage II (70 pts) - median 72 months, stage III (25 pts) median 26.0 months. Significant prognostic parameters for poor survival were: age at transplant over 60 years (p under 0.001), IgA typ of monoclonal immunoglobulin (p=0.036), renal impairment with serum creatinine at diagnosis over 2 mg/dL (p=0.007), no achievement of CR after ASCT (p under 0.001). The status of disease before ASCT and type of maintenance therapy after transplant (alone interferon (IFN), IFN + dexamethasone, 4 cycles of chemotherapy CED and after it IFN) did not significantly affect OS after ASCT. *Conclusion.* In our group of patients the survival after ASCT correlated with the stage according to ISS, age, clinical response after transplant, type of paraprotein and renal impairment at diagnosis. The most significant parameters for better survival of MM pts after transplant are: age under 60 years at transplant, no ISS stage III at diagnosis and achievement of CR after transplantation.

0226

IDENTIFICATION OF NOVEL GENE EXPRESSION SUBGROUPS IN MULTIPLE MYELOMA USING UNSUPERVISED CLUSTER ANALYSIS

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Background. Accumulation of malignant plasma cells in the bone marrow is a hematological malignancy referred to as multiple myeloma (MM). Heterogeneity in clinical presentation and molecular markers strongly suggest that MM is a conglomerate of diseases with different molecular mechanisms. This is the most likely explanation for heterogeneous response of myeloma patients to therapeutic approaches such as high-dose therapy, conventional chemotherapy or the new agents bortezomib and thalidomide/Imids. Future development of effective therapies and specific strategies to overcome therapy-resistance will only be possible if we are able to recognize and understand the biological subtypes of myeloma. Therefore, there is a continuous need to improve the molecular classification of MM. *Aims.* Our hypothesis is that unsupervised cluster analysis of gene expression profiles will provide an improved molecular classification of myeloma patients compared to cytogenetics alone. The aim of this study is to test this hypothesis. *Methods.* Expression data of 173 newly diagnosed, untreated myeloma patients previously reported by Tian *et al.* (NEJM 2003,349:2483-2494) were downloaded from the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo), accession number GDS531. The dataset contains expression data that were obtained using Affymetrix U95Av2 arrays and were normalized using the method of global scaling, provided in the Affymetrix MAS5.0 software. Unsupervised hierarchical cluster analysis was performed with complete linkage and Euclidean distance as similarity metric, using the Omniviz package. Supervised analyses were performed with the use of SAM software. Cluster-specific gene lists obtained using the SAM method were imported in EASE v2.0. Based on available annotations in Gene Ontology and the GenMAPP database we determined which pathways or processes were statistically overrepresented in the cluster-specific gene lists. Correction for multiple testing was performed using the Benjamini-Hochberg method in EASE v2.0. *Results.* Unsupervised cluster analysis defined ten clusters based on overall correlation. Clusters displayed unique, non-overlapping gene expression signatures. Six of these clusters have not been described before. Three clusters corresponded to recurrent 14q32 translocations: t(4;14), t(11;14) and t(14;16)/t(14;20). One cluster appears to represent polyclonal plasma cell preparations. One of the novel clusters displayed specific expression of WNT10B, BIK and CST6 combined with STAT1 downregulation. A novel subgroup of t(11;14) patients was identified as a particularly distinct cluster lacking expression of TNFRSF7/CD27 and specifically expressing RBP1 and RAB33A. Samples from patients lacking myeloma-related bone lesions mainly grouped together in only three clusters. FRZB downregulation was specifically associated with these three clusters. Analysis of cluster specific gene signatures identified expression of WNT10B, bone morphogenetic protein 4, and osteopontin as specific events in subgroups with low bone-disease frequencies. *Summary/Conclusion.* Using gene expression profiling we have identified a novel subgroup of t(11;14) myeloma patients and a new genetic cluster in which only 37% of the patients were diagnosed with bone disease. The unsupervised nature of our analysis has proven a powerful method for the classification of MM patients, showing improved discriminating capacity.

0227

EFFICACY OF SINGLE-AGENT BORTEZOMIB VERSUS THALIDOMIDE IN PATIENTS WITH RELAPSED OR REFRACTORY MULTIPLE MYELOMA: A SYSTEMATIC REVIEW

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Aim. To perform a systematic review of the efficacy of monotherapy with bortezomib versus thalidomide in patients with relapsed or refractory multiple myeloma. *Methods.* Scientific literature published in English from 1966 to June 2005 (MEDLINE, EMBASE, Cochrane library), publication reference lists, Janssen-Cilag Pty Ltd data-on-file, and abstracts from recent multiple myeloma conferences were reviewed. Prospective studies containing at least a single arm of either treatment group with n ≥30 and using continuing or variable thalidomide dosing were included. Studies adding dexamethasone for non-responders were excluded. Outcomes were analysed on an intent-to-treat basis. Statistical pooling was performed where possible for the primary outcome of response rate, defined by a serum M-protein reduction ≥50% (A) and by strict (e.g. European Bone Marrow Transplant, EBMT) criteria (B), and for the secondary outcomes of overall survival and progression-free survival. *Results.* One bortezomib (n=333, APEX, NEJM 2005, 352; 2487-98) and 15 thalidomide (n=1007) studies were included. Patient baseline characteristics including age, gender, IgG:IgA, disease duration and β-2 microglobulin (β2M) were well matched, except that 48% of bortezomib patients had received prior thalidomide. On an intent-to-treat basis, the overall estimate for response rate (A) was 53% for patients receiving bortezomib versus 32% for thalidomide (p<0.001, n=10 studies). For response rate (B) the estimate was 36% for patients receiving bortezomib versus 22% for thalidomide (p<0.001, n=4 studies). One-year overall survival was 80% for patients receiving bortezomib versus 63% for thalidomide (p<0.001, n=6 studies). Due to differences in disease monitoring and definitions of progression, it was not possible to compare results for progression-free survival. *Conclusion.* Bortezomib was associated with a significantly higher response rate and a greater proportion of patients achieving one-year overall survival than thalidomide in patients with relapsed or refractory multiple myeloma, despite 48% of bortezomib treated patients having received prior thalidomide.

0228

BORTEZOMIB IN RELAPSED MULTIPLE MYELOMA: RESPONSE RATES AND DURATION OF RESPONSE ARE INDEPENDENT OF CHROMOSOME 13Q

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Background. Presence of a chromosome 13q-deletion confers a poor prognosis to patients (pts) with multiple myeloma (MM), even in the context of intensive treatment programs and thalidomide. Bortezomib is the first compound of a new class of agents *the proteasome inhibitors* showing activitiy in relapsed and chemotherapy-refractory MM. Results of SUMMIT and APEX trials suggested that bortezomib is active in MM with previously recognized unfavorable prognostic factors. *Aims.* To study the activity of bortezomib in relapsed MM and potential associations with prognostic factors (standard clinical parameters and chromosomal aberrations: deletion of chromosome 13q14 [del(13q14)], 14q-translocations [t(14q32)], gain of 1q21). *Patients and Methods.* We evaluated 51 consecutive pts with relapsed/refractory MM (median number of prior therapies: 3; 92% had high-dose, pulsed dexamethasone, 71% thalidomide, 43% high-dose therapy; median time from first line therapy to bortezomib, 4.8 years). Treatment consisted of single agent bortezomib according to the standard regimen (1.3 mg/m^2 on days 1, 4, 8, 11; q21 days). Chromosomal abnormalities were determined by means of interphase FISH. *Results.* Similar response rates to bortezomib were observed in pts with del(13q14) (13 of 26 pts = 50%) and with normal chromosome 13q (15 of 25 pts = 60%) (p=0.34). Of note, rates of CR/nearCR were also not different between the two patient populations (23% vs. 16%). Moreover, median duration of response was 10.4 months in pts with del(13q14) compared with 9.3 months in pts with normal 13q-status (p=0.29). Only those pts with del(13q14) who did not show a response to bortezomib experienced a rapidly progressive clinical course leading to overall shortened survival. For an improved identification of such pts, additional parameters were tested. In a subset of pts, analyses for gain of 1q21 (CKS1B gene) were performed. Among 10 pts with simultaneous del(13q14) and gain of 1q21, 8 failed to respond to bortezomib, and their median survival was only 3.3 months. We also observed that pts with low serum levels of albumin had a poor outcome after bortezomib. Thus, pts not benefiting from single-agent bortezomib were characterized by the combined presence of a del(13q14) and low serum albumin (median survival 5.3 months). β-2-microglobulin, however, was not important for treatment outcome after bortezomib. Finally, 3 pts were found to have a t(4;14)(p16;q32) in addition to a del(13q14), and all of them had a > 50% reduction of their paraprotein after bortezomib. *Conclusion.* Our results indicate that bortezomib has good clinical activity in MM patients with high-risk cytogenetic features. The simultaneous occurrence of a del(13q14) with gain of 1q21 and/or low serum albumin allows for the identification of pts not benefiting from single-agent bortezomib. It is suggested to evaluate bortezomib combinations in such pts.

0229

INTEGRATIVE GENOMIC ANALYSIS OF MULTIPLE MYELOMA PATIENTS WITH 13Q DELETION SUGGESTS A ROLE OF CHROMOSOMAL ABERRATIONS IN THE TRANSCRIPTIONAL FINGERPRINT

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Background. The chromosome 13 deletion [del(13)] represents one of the most frequent chromosomal alterations in multiple myeloma (MM), characterizing almost 50% of the patients. Several groups have reported an unfavorable prognostic role for del(13) in MM although, according to some authors, the prognostic value of del(13) should not be considered *per se* but has to be related to the ploidy or to the main chromosomal translocations involving 14q32 locus. *Aims.* To better characterize the biology of del(13), the purpose of the present study was to provide a comprehensive analysis of the transcriptional profiles and the molecular features associated with del(13) on MM patients. *Methods.* The transcriptional profiles of 90 MM newly diagnosed MM patients have been generated from highly purified plasma cells by means of high-density oligonucleotide arrays (Affymetrix GeneChip U133A) and subsequently analysed using unsupervised and supervised approaches (two-dimensional hierarchical clustering and SAM, respectively). Chromosomal regions with modulation of the gene expression signals have been identified using a non-parametric model-free statistical method (LAP, locally adaptive statistical procedure). The aneuploidy status was evaluated by fluorescence *in situ* hybridization (FISH) analyses using a Trisomy Index recently proposed (Wuilleme S. *et al.,* Leukemia, 2004). Genome wide profiling data for 10 MM samples have been generated on high-density SNP arrays (Affymetrix GeneChip Human Mapping 10k Xba 142 2.0 arrays) and analysed to investigate copy number alterations. *Results.* The differential expression of 87 transcripts (specific for 67 genes), all of them downregulated in del(13)+ group, distinguished del(13)+ from del(13)- MM cases; forty-four genes were localized along the whole chromosome 13, 7 on chromosome 11 and 4 on chromosome 19. The majority of the identified genes resulted involved in translational pathways. In addition, we identified the presence of the putative tumor suppressor gene *RFP2*, mapping at 13q14.3 within the minimally deletion region. An integrative genomic approach, based on the regional analysis of gene expression data, allowed detecting novel chromosomal regions whose modulation in global expression levels could differentiate the del(13)+ patients. In particular, we identified the upregulation of the 1q42 region and the downregulation of the 19p region and of almost the entire chromosome 11. To better clarify these findings, we investigated the specific chromosome regions by FISH, showing a strong relationship between del(13)+ and either the presence of 1q21-1q42 amplifications (p=6×10^{-4}) or the absence of chromosome 11 trisomy (p=5×10^{-4}). Finally, the genome wide profiling of 10 MM patients included in our study confirmed the patterns observed by FISH. *Conclusions.* By combining integrative genomic approaches and FISH analyses, we evidenced that the presence of the chromosome 13 deletion in MM is specifically associated with distinct types of chromosomal aberrations, which may be responsible for the transcriptional differences between del(13)+ and del(13)- patients.

0230

EFFICACY AND SAFETY OF MELPHALAN/ARSENIC TRIOXIDE/ASCORBIC ACID COMBINATION THERAPY (MAC) IN PATIENTS WITH RELAPSED/REFRACTORY MULTIPLE MYELOMA: A PROSPECTIVE, MULTICENTER, PHASE II, SINGLE-ARM STUDY

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Background. Multiple myeloma (MM) is an incurable B-cell malignancy, and nearly all patients develop resistant disease. Most patients develop renal insufficiency, which is associated with a poor survival. Thus, it is imperative to explore new therapeutic options that can improve their renal function and prognosis. Arsenic trioxide (ATO) is an active anti-MM agent. In preclinical MM studies, the addition of ATO to the cytotoxic agent melphalan overcomes resistance to this alkylating agent. Moreover, ascorbic acid (AA) enhances the cytotoxic effects of ATO. Importantly, a small pilot clinical study of melphalan, ATO, and AA (MAC) therapy for relapsed and refractory MM patients has shown that this combination is well tolerated, with significant durable responses as well as improved renal function for the patients with baseline azotemia. *Aims.* The primary objectives were to determine response rate, time to progression, and safety and tolerability of MAC therapy in a larger multicenter trial. The secondary objectives were to analyze time to response, progression-free survival, overall survival, and the effects of MAC therapy on renal function. *Methods.* Patients with relapsed or refractory MM

received melphalan (0.1 mg/kg PO), ATO (0.25 mg/kg IV), and AA (1 g IV) on days 1-4 of week 1, ATO and AA twice weekly on weeks 2-5, and rest during week 6 of cycle 1; melphalan on days 1-4 and ATO and AA twice weekly on weeks 1-5, and rest during week 6 of cycles 2-6. *Results.* Patients (N = 65) had failed a median of 4 (range, 1-8) prior therapies, including melphalan, bortezomib, thalidomide/lenalidomide, glucocorticosteroids, and peripheral stem cell transplantation. Objective responses were observed in 31 (48%) patients, including 2 complete (CR), 15 partial (PR), and 14 minor responses (MR). The median time to progression, time to response, and overall survival were 7 months (0-25+ months), 2 months (2-6 months), and 19 months (2-27+ months), respectively. Notably, of the 23 patients who had elevated baseline serum creatinine (SCr) levels, 17 (73%) showed improvement in renal function. Grade 3 or 4 anemia and/or neutropenia occurred in 3 patients and in 1 patient, respectively. Common grade 3 or 4 nonhematologic adverse events were fever/chills (15%), pain (8%), and fatigue (6%). Two patients had single occurrences of prolonged QTc interval (498 and 502 msec) resulting in a brief delay in ATO administration, but continued ATO dosing was not accompanied by any further episodes of QTc prolongation. One patient developed unstable bradycardia without a prolonged QTc following the first ATO infusion and was removed from the study. *Conclusions.* The MAC combination regimen was an effective treatment for patients with relapsed or refractory MM, producing objective responses in half of the patients in this heavily pretreated group. Patients with renal insufficiency at baseline showed improvements in renal function with MAC therapy. The MAC regimen was well tolerated, with relatively few grade 3 or 4 hematologic adverse events or cardiac events. These results show that the MAC combination regimen is an effective and well-tolerated new therapeutic option for patients with relapsed or refractory MM.

0231

INSULIN-LIKE GROWTH FACTOR 1 (IGF-1) IS OVEREXPRESSED IN MULTIPLE MYELOMA PLASMA CELLS (PC) AND REGULATES THE EXPRESSION OF THE IGF-1 RECEPTOR

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*Background.*IGF-1 plays an important role in regulating cell proliferation, differentiation, apoptosis, and transformation. Recent studies have shown that IGF-1 is an important survival and growth factor in multiple myeloma (MM). Moreover, IGF-1 down-regulates IGF-1R expression at the transcriptional level, by autocrine or paracrine mechanism. *Methods.* The expression of IGF-1 and IGF-1R was evaluated by Real-time PCR in a series of 53 newly diagnosed MM patients primarily treated with thalidomide and dexamethasone. For each patient, we isolated the CD138+ cell fraction from bone marrow (BM) sample at diagnosis and, in 24/53 patients for whom material was available, also at the end of induction therapy; both CD138+ and CD138- cell fractions were studied. A pool of donors was used as calibrator. The Mann-Whitney and the Spearman Rank Correlation tests were applied for statistical analysis. *Aim.* Correlate the expression of these genes with presenting karyotypic features of MM patients and evaluate their relationship with response to therapy. *Results.* Both neoplastic PC and CD138- cell fractions expressed a markedly high levels of IGF-1 (median 145.01 and 3.07, range 0.13-1089.92 and 0.02-103.25, respectively), and low levels of IGF-1R (median 0.76 and 1.77, range 0.04-10.78 and 0.21-13.55, respectively). Expression data resulted very scattered; as a consequence, no differences could be highlighted in IGF-1 and IGF-1R expression, with respect neither to the most common presenting clinical features, nor to the presence of t(4;14) and del(13). According to response to induction therapy (39 responsive and 15 non responsive patients) again, no relationship between the expression of IGF-1 and IGF-1R was pointed out. In order to look for indications of an autocrine negative-feedback regulatory mechanism, we looked for correlations between the expression values of IGF-1 and IGF-1R; however we could not detect any significant inverse correlation, in any analyzed fraction. On the contrary, a significant inverse correlation was highlighted between the CD138+ expressed IGF-1 and the CD138- expressed IGF-1R (p= 0.01, r= -0.33), thus suggesting a possible paracrine effect of PC-produced IGF-1 exerted on CD138- cells, which resulted more enhanced when analysing patients subgroups not harbouring t(4;14) or del(13) (p<0.0001, r= -0.59 and p<0.0005, r= -0.59, respectively). Conversely in t(4;14)+ patients, the correlation between IGF-1 and IGF-1R expression become significantly positive (p= 0.03, r= 0.64). After induction therapy, a median IGF-1R increase was observed among CD138+ samples (0.67 vs. 1.3, p=0.03); patients not harbouring del(13) and t(4;14) showed the most relevant increase (0.6 vs. 1.58, p=0.03 and 0.53 vs.1.13, p=0.0005, respectively). *Conclusions.* Our preliminary study confirmed the involvement of IGF-1/IGF-1R pathway in MM pathogenesis; we suggested a paracrine effect of PC-produced IGF-1 on CD138- cells, which seemed to act only in responding patients. The ability to efficiently regulate IGF-1R expression may thus have an important prognostic value. Moreover, a different regulation of IGF-1/IGF-1R pathway may exist between different genetic subtypes. The study of post transduction modifications of the IGF-1R will be needed, in order to get more insight into the relationship between the IGF-1 and IGF-1R expressions and IGF-1R activation.

0232

AUTOIMMUNITY IS ASSOCIATED WITH BETTER SURVIVAL IN MULTIPLE MYELOMA

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Background. In Western countries, multiple myeloma (MM) is the second most common hematopoietic malignancy after non-Hodgkin lymphoma. MM remains yet an incurable B-cell malignancy with a median survival of 3 to 4 years. Autoimmunity is associated with improved outcome in patients with certain tumors suggesting that host-related immune response plays an important role in the pathogenesis. Given the association between MM and certain autoimmune diseases, documenting the impact of autoimmunity on risk of MM survival might provide clues to exploiting the host immune reaction for enhanced treatment strategies involving host immunity. *Aims.* To assess the prognostic significance of autoimmunity in patients with MM. *Methods.* Records on 10,557 population-based MM patients reported to the Swedish (1964-1998) and Danish (1977-1997) Cancer Registries were linked to the nationwide Inpatient Registries to capture hospital records including data on 32 defined autoimmune disease and the Cause of Death Registries to retrieve mortality data. Using logistic regression models adjusted for gender, age, and country, we defined risk of MM mortality in relation to presence/absence of any autoimmune disease and for categories of autoimmune conditions: (Group A) autoantibodies (AAB) with systematic involvement, (Group B) AAB with organ-specific involvement, and (Groups C) no AAB. We also evaluated risk of MM mortality for individual autoimmune conditions. Based on the expectation that secular trends in MM and autoimmune disease diagnostics/treatment could introduce heterogeneity we explored models stratified by calendar-period. Significant effect-modification by calendar-period was observed so results are presented by the approximate mid-point of year of MM diagnosis (<1987 vs. ≥1987) strata. *Results.* In the first calendar-period (<1987) we observed overall significantly decreased risk of MM mortality among persons with presence (n=578) of any autoimmune disease (OR=0.44, 95% CI 0.25-0.79). When we fit models by the 3 autoimmune disease categories, we observed decreased MM mortality for each: Group A (OR=0.34, 95% CI 0.15-0.75), Group B (OR=0.71, 95% CI 0.25-1.97), and Group C (OR=0.32, 95% CI 0.13-0.82), although only Groups A and C reached formal significance. The Group A effect was driven by the conditions rheumatoid arthritis and polymyositis/dermatomyositis; and the Group C effect was driven by conditions including ankylosing spondylitis, rheumatic fever, sarcoidosis, and polymyalgia rheumatica. In the second calendar-period (>1987), we found no statistical associations between MM mortality and presence of any autoimmune disease (OR=1.20, 95% CI 0.92-1.56), Group A (OR=1.25, 95% CI 0.78-2.01), Group B (OR=1.39, 95% CI 0.87-2.21), Group C (OR=0.97, 95% CI 0.66-1.43), or individual autoimmune conditions. Estimates were similar when analyses were restricted to autoimmune diseases documented only prior to MM diagnosis (n=520). *Summary/Conclusions.* The decreased mortality among MM patients diagnosed in the first calendar-period with certain autoimmune diseases is intriguing and provides support for the role of host-related immunity as an anti-tumor agent in MM therapy. The observed protective effect in the first (1964-1986), but not in the second (1987-1998), calendar-period might reflect variations in diagnostic procedures, clinical management, and/or treatment strategies for autoimmune disease and/or MM. Future studies are needed to clarify underlying mechanisms of our findings.

0233

BORTEZOMIB AND HIGH DOSE MELPHALAN: A NEW CONDITIONING REGIMEN BEFORE AUTOLOGOUS STEM CELL TRANSPLANTATION FOR MULTIPLE MYELOMA

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Background. The achievement of Complete Response (CR= disappearance of M component by immunofixation) or Very Good Partial Response (VGPR= >90% of M component reduction) is the main prognostic factor for survival after Autologous Stem Cell Transplantation (ASCT) in Multiple Myeloma (MM). High Dose Melphalan (HDM) (200mg/m^2) is the recommended conditioning regimen before ASCT. However, the rate of CR+VGPR is only 40% to 50%. Bortezomib (BOR) has demonstrated a significant activity in relapsed/refractory patients, a synergistic effect with Melphalan (MEL) and a lack of haematological toxicity. *Aims.* The combination of BOR and HDM was a logical approach to improve the rate of CR+VGPR after ASCT. *Methods.* Between June 2005 and February 2006, 25 patients with stage II or III DS MM have been enrolled to receive an ASCT conditioned with both BOR and HDM. BOR (1 mg/m^2/d) was delivered on days - 6, - 3, + 1 and + 4, MEL (200 mg/m^2) was administered on day - 2, and blood stem cells (median 3,3×10^6 CD34/kg) were infused on day 0. The dose of BOR was initially started to 1.3 mg/m^2/d for 3 patients but was lowered because of cardiac effects. *Results.* The main characteristics of the 25 patients were: median age = 56 years; M component= IgG in 17 cases and IgA in 4 cases; chromosome 13 deletion in 6 cases on 13 assessable; β-2 microglobulin >3 mg/L in 10 cases. Fourteen patients were in first line therapy. No patient was in CR before ASCT, 3 were in VGPR and 4 were in first partial response (PR= > 50% of M component reduction). Seven had only a minimal response (MR) after VAD regimen. Four patients had already received 2 or more prior treatments before ASCT. Seven patients were in MR or with a progressive disease (PD) after a first course of classical HDM (1 patient was progressive after a tandem intensification). BOR was not found to increase the haematological toxicity observed after HDM. The median duration of neutropenia (< 0.5×10^9/L) and thrombocytopenia (< 20×10^9/L) was 7 and 2 days respectively. Extra-haematological toxicities were limited: grade 3/4 mucositis in 4 cases, erythrodermy in 6 cases and cardiac arrhythmia in 3 cases. Cutaneous reactions were mainly reported in association with glycopeptide antibiotics. No toxic death was observed. Thirteen patients were assessed for early response at 3 months after ASCT. Four patients (31%) achieved a CR and 6 (46%) a VGPR. Two patients were non responders. Among the 4 patients receiving a second ASCT, 2 CR and 1 VGPR were observed after BOR+HDM at 3 months. *Conclusions.* These preliminary results strongly suggest that BOR (1 mg/m^2 ×4) and HDM is a safe and highly effective conditioning regimen in MM, requiring further investigations. For the 13 patients assessed at 3 months, the rate of CR+VGPR was 77%.

0234

ENOXAPARIN OR ASPIRIN FOR THE PREVENTION OF RECURRENT THROMBOEMBOLISM IN NEWLY DIAGNOSED MYELOMA PATIENTS TREATED WITH MELPHALAN AND PREDNISONE PLUS THALIDOMIDE OR LENALIDOMIDE

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Background. Venous thromboembolism (VTE) is a common complication in cancer patients. The risk is particularly high after surgery, during chemotherapy and in association with central vein catheters. Aggressive antitumor therapy with thalidomide or lenalidomide increases the risk of thrombosis. The underlying mechanisms are poorly understood, but these therapeutic agents induce vascular damage. The risk of thrombosis is higher for patients receiving thalidomide at diagnosis in comparison with those treated at relapse. Low-molecular weight heparin is considered the standard prophylaxis in these patients. Low-intensity warfarin and aspirin have also been used. Patients received melphalan, prednisone (MP) alone; or MP plus thalidomide (MPT) without any anticoagulant prophylaxis; or MPT and enoxaparin; or MP plus lenalidomide (Revlimid®) (RMP) and aspirin. *Aims.* We evaluated the efficacy and safety of enoxaparin or aspirin in the prevention of VTE, in newly diagnosed myeloma patients. *Methods.* In the MP group, no patient received anticoagulant prophylaxis. In the MPT group, MP was combined with Thalidomide (Pharmion Ltd., Cambridge, UK), no anticoagulant prophylaxis was administered until December 2003. In a preliminary analysis, an high incidence of thrombosis was observed, therefore the protocol was amended and enoxaparin at 40 mg per day was introduced as prophylaxis and delivered subcutaneously during the first four cycles of MPT therapy. In the RMP group, all patients received aspirin 100 mg once a day continuously until any sign of relapse or progressive disease. The time to occurrence of the first thromboembolism was calculated from the start of chemotherapy. *Results.* In the MP group, VTE was reported in 2 of the 144 patients; in the MPT group, symptomatic deep-venous thrombosis, pulmonary embolism, or both occurred in 12 of the 65 patients who did not receive any anticoagulant prophylaxis. Thromboembolism was observed in 4 of the 78 MPT patients who received enoxaparin prophylaxis; in RMP group, one of 50 patients, who received aspirin, experienced pulmonary embolism. Median time for VTE was 4 months in the MP group, 3 months for MPT with and without anticoagulant prophylaxys. In the RMP group, the only episode of thromboembolism occurred after 1 months from start of therapy. In comparison with MP, the hazard ratio for recurrent VTE in the MPT group without any prophylaxis was 14.3 (95% CI, 3.2 - 64.3; p<0.0001); in the MPT group with enoxaparin it was 3.76 (95% CI, 0.69 - 20.52; p=0.11); in the RMP group with aspirin it was 1.72 (95% CI, 0.15 - 19.8; p=0.67). No significant interactions between treatment group and risk factors were detected. No serious bleeding was observed during both aspirin and enoxaparin prophylaxis. *Conclusion.* MPT with enoxaparin and RMP with aspirin were safe and equally effective in reducing the risk of recurrent VTE to levels observed in patients who received oral MP only.

0235

INTERMEDIATE-DOSE MELPHALAN (100MG/M^2), THA5LIDOMIDE, DEXAMETHASONE AND STEM CELL SUPPORT IN PATIENTS WITH REFRACTORY OR RELAPSED MYELOMA

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Background. Combination approaches of new drugs with conventional therapies have increasingly been adopted as savage or even first-line treatment for multiple myeloma. High-dose or dose-intensive i.v. melphalan followed by hematopoietic cell support induced higher response rates and improved outcome compared to conventional oral melphalan in several randomized trials. These findings formed the rational for the combination of both bortezomib and thalidomide with intermediate-dose melphalan (100mg/m^2) as conditioning regimen prior to autologous hematopoietic cell infusion. No data are available on the use of this combination as conditioning regimen in the transplant setting. *Aims.* We assessed the safety, tolerability and response rate of intermediate-dose melphalan, Velcade, thalidomide and dexamethasone followed by stem cell support in refractory or relapsed multiple myeloma (MM) patients. *Methods.* Twenty-six advanced myeloma patients were treated with melphalan at 50 mg/m^2 and bortezomib at 1.3 mg/m^2 on days -6 and 3 associated with thalidomide at 200 mg and dexamethasone at 20 mg on days 6 through -3 (MVDT), followed by hematopoietic cell support on day 0. *Results.* Between September 2004 and December 2005, 26 patients with relapsed or refractory MM were enrolled in the study. Median time from diagnosis was 48,5 months (range 2,3-142 months). All patients were induced with standard autologous transplants. Moreover, 14 (54%) were treated with a combination of thalidomide and dexamethasone and 13 (50%) with a second autologous transplant as salvage treatments. Objective responses occurred in 17 of 26 patients (65%), including one complete remission (CR 3%), 3 near complete remissions (nCR, 11%) and 2 very good partial response (VGPR 7%); 3 patients (10%) showed minimal response. Six patients (23%) showed no response (NR) and no patients showed progressive disease (PD). Interestingly, of 5 patients who had previously progressed while on thalidomide and prednisone, 1 reached nCR, 2 PR and 1 MR. After a median of 9 months (range 1-16), 7 patients (27%) were alive in remission, 15 patients (58%) relapsed, 4 patients (15%) died from progression disease and one patient from infective toxicity. Median progression-free survival for all patients was 6 months (range, 1 to 16 months). Response rate was higher than that induced by the previous line of treatment in 12 patients (46%); response duration was longer than in 6 patients (30%). Grade 3 thrombocytopenia developed in 46% of patients, grade 4 in 54%. Fourty-two% of

patients developed grade 3 anemia, 38% grade 4, whereas all patients showed grade 4 neutropenia. Five patients (19%) showed grade 1-2 neurologic toxicity, 1 patient grade 3. Infections consisted of pneumonia in 9 patients (35%), fatal for one patient and neutropenic fever (12%). Infections required iv broad spectrum antibiotic therapy in 50% of the patients. *Conclusion.* MVTD showed encouraging activity with manageable toxicity and represents a promising treatment for advanced myeloma patients.

0236

THALIDOMIDE-DEXAMETHASONE VS THALIDOMIDE-DEXAMETHASONE AND PEGYLATED LIPOSOMAL DOXORUBICIN: A CASE-MATCHED STUDY IN PATIENTS WITH ADVANCED MULTIPLE MYELOMA

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Background. Thalidomide alone or in combination with dexamethasone and/or chemotherapy is the most extensively used compound in the treatment of relapsed/refractory multiple myeloma (MM). However, which thalidomide-based regimen is more effective and less toxic is still unknown. Recently we demonstrated that the combination of thalidomide, dexamethasone and pegylated liposomal doxorubicin (ThaDD) leads to high rate and high quality of response (ORR=92%; CR/nCR= 32%) with a PFS of 47% and OS of 65% at 2 years. *Aims.* In the present study we compared ThaDD with the combination thalidomide-dexamethasone (T-D), frequently used in advanced MM patients as salvage therapy. *Methods.* A total of 47 relapsed/refractory patients treated ThaDD was compared with a control group of 47 pair mated matched patients for age, serum β2-microglobulin, previous chemotherapy and high-dose therapy. ThaDD regimen consisted of thalidomide 100 mg/day continuously, dexamethasone 40 mg on days 1-4 and 9-12, pegylated liposomal doxorubicin 40 mg on day 1 every 28 days. T-D regimen consisted of thalidomide 100 mg/day continuously and dexamethasone 40 mg on days 1-4 repeated monthly. Both groups included a lot of elderly patients, who had received 3 or more prior chemotherapy regimens and who had undergone stem cell transplantation. *Results.* ThaDD significantly increased overall response rate in comparison with T-D (92% vs 63.5%; p=0.008) and, importantly, induced significantly better quality of response (≥PR 75.5% vs 59.5%, p=0.077; ≥VGPR 36% vs 15%, p=0.018; CR/nCR 30% vs 10.5%; p=0.021). Compliance to therapy was satisfactory in both groups of patients and grade 3-4 neurological toxicity were limited (4.2% in patients treated with ThaDD vs 2.1% in those receiving T-D). On the contrary, grade 3-4 hematological toxicity (32% vs 0; p<0.0001), grade 3-4 infections (23% vs 0; p<0.0001) and vascular events (12.8% vs 6.4%; p=0.293) were more frequent in patients treated with ThaDD although no deaths were related to these complications. The rate of infections decreased below 10% when ciprofloxacin was added in the ThaDD regimen. The median PFS was significantly longer in ThaDD group (22 months vs 11.5 months, 36% vs 13% at 3 years; p=0.0008) as well as median EFS (21 months vs 11.5 months, 28% vs 13% at 3 years; p=0.0077) and OS (NR vs 23.5 months, 52% vs 26% at 3 years; p=0.0511). *Conclusions.* ThaDD, as salvage therapy for MM, regimen is superior to the combination thalidomide-dexamethasone since it induces a significantly higher and better quality response rate than T-D and this translates into a significantly better survival measures. The incidence of infections and deep venous thrombosis are more frequent in ThaDD group but they result manageable with adequate prophylaxis. We believe that ThaDD combination could be a valid candidate for comparison with bortezomib- or lenalidomide-based regimens in order to identify the optimal salvage therapy in advanced MM.

0237

DYSFUNCTION OF TOLL-LIKE RECEPTORS: A POSSIBLE IMPLICATION IN THE PATHOGENESIS OF IMMUNODEFICIENCY IN MULTIPLE MYELOMA

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Background. Immune paresis, renal failure, neutropenia and antimyeloma therapy can combine to cause severe immunodeficiency in multiple myeloma (MM). Thus, infections are a major cause of death in MM. There is limited information for the possible role of innate immunity in the pathogenesis of immunodeficiency in MM. Innate immunity provides a first line of host defence against infection through microbial recognition and killing while simultaneously activating a definitive adaptive immune response. Innate immune detection of pathogens relies on specific classes of microbial sensors, such as Toll-like receptors (TLRs). TLRs are principal mediators of rapid microbial recognition and function mainly by detection of structural patterns that do not exist in the host. *Aims.* The aim of this study was to evaluate the expression and function of TLRs in newly diagnosed MM. According to our knowledge, such information is not available in the literature. *Patients and Methods.* Twenty-two patients with MM at diagnosis (13M/9F; median age 68 years), 2 patients with MGUS and 11 healthy, age- and gender-matched controls were studied. Five patients had stage 1, 9 stage 2 and 8 stage 3 myeloma, according to ISS. After the collection of peripheral blood, mononuclear cells (PBMCs) were isolated by Ficoll centrifugation (Histopaque-1077; Sigma-Aldrich). These cells were measured for the expression of TLRs (antibodies from eBioscience) using fluorescence activated flow cytometry (FC 500, Beckman Coulter). In addition, 1×10^6 cells/mLwere cultured in 5% FCS 1% pen/strep RPMI in the presence or absence of various TLR ligands and supernatants collected after 20h. These were examined for the presence of inflammatory cytokines (tumor necrosis-α, TNF-α; and interleukin-6, IL-6) by ELISA (Becton Dickinson). *Results.* We found that although patients with MM express TLRs in PBMCs, their response to certain TLR ligands is defective when compared to healthy controls. TLR2, TLR4 and TLR6 of PBMCs of healthy controls reacted normally to the presence of their respective ligands PAM3CYS (gram positive and negative bacterial), LPS (gram negative) and FLT-1 (gram positive), producing median value of 2.2 ng/mL of TNF-α (range: 1-3 ng/mL), while their action in patients with MM was significantly reduced (median value of TNF-α: 200 pg/mL; range 100-500 pg/mL; p<0.001). On the contrary, TLR7 and TLR8 from MM reacted normally to their ligand R-848 (Imiquimob) to secrete high levels of TNF-α (median value for patients and controls: 4.3 and 4.5 ng/mL, respectively; p=NS). NOD1, another pattern recognition receptor that recognizes bacterial peptidoglycans also reacted normally in MM patients. Similar observations have been made for the expression of IL-6. Our preliminary analysis showed that there was no difference in terms of TLRs function between MGUS patients and controls and between myeloma patients of different disease stages. *Conclusions.* There is a significant defect in TLR function in patients with MM, especially of these involved in immunity against bacterial infections. Thus the immune system fails to receive early priming signal which may contribute to the increased infections observed in MM. The restoration of function of TLRs to their normal levels has the potential to improve bacterial immunity in MM patients.

0238

CLINICOPATHOLOGICAL CORRELATES OF PLASMA CELLS CD56 (NCAM) EXPRESSION IN MULTIPLE MYELOMA

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It was shown that absence of CD56 on malignant plasma cells (PCs) is hallmark of plasma cell leukemia (PCL), special subset of multiple myeloma (MM) (Leukemia 1998; 12: 1977) and may play role in pathogenesis of central nervous system MM (Br J Haematol 2005; 129: 539). There was also found that expression of CD56 correlates with presence of osteolysis in MM and distinguishes MM from MGUS and lymphomas with plasmacytoid differentiation (Am J Pathol 2002; 160: 1293). Aim of this study was to evaluate intensity of CD56 expression on bone marrow (BM) myelomatous PCs and to assess clinical correlations. The study group consisted of 186 MM patients (100M 86F, median age 63, range 32-89yr ;28 at stage I, 43-II, 115 -III; 137 had osteolysis; monoclonal protein IgG was in 118 patients, IgA-45, IgD-1, IgM-2, Bence Jones'18, NS-2) and 16 PCL patients. Controls were 10 healthy subjects. Immunophenotyping was done on freshly collected BM samples using triple staining combination of CD138/CD56/CD38 monoclonal antibodies analysed by flow cytometry (Cytoron Absolute and FACSCalibur-Becton Dickinson). Plasma cells were identified as cells showing high-density expression of CD38 and CD138 (syndecan-1). Antigen expression intensity was calculated as relative fluorescence intensity (RFI) and for direct quantitative analysis the QuantiBRITE test was applied. Mean channels of phycoerythrin fluorescence were defined and antibody bounding capacity (ABC) was then calculated using QuantiCALC software. *Results.* In 128 patients (69%) PCs showed CD56 expression. Out of all CD38++/CD138+ BM cells mean proportion of PCs with CD56 expression, was 83±20%, median 93%. RFI values ranged from 7,6 to 27,4 in particular patients (18,0±4,5, median 17,8) and the number of CD56 binding sites (ABC) on MM plasma cells ranged from 2255 to 58469 (14199±15038, median 8866). A correlation was found between RFI and ABC values (r=0,76; p<0,001)). In 58 MM patients considered as CD56 negative myeloma mean proportion of all BM CD38++ cells with CD56 expression was 4,8±4,2%, median 3,5%. A correlation was found between proportion of all BM CD38++ cells with CD56 expression and ABC (r=0,60) and RFI (r=0,62) indices (p<0,001). Normal PCs did not express CD56. Osteolytic lesions were found in 80% of CD56+ MM patients and in 60% of patients with CD56 negative myeloma. When comparing other clinical and biological disease characteristics e.g. monoclonal protein isotype, b2M, LDH, stage of disease, calcium, creatinine, response to chemotherapy, survival time of CD56 positive and CD56 negative cases, no significant differences were found. Of 16 PCL cases 8 showed CD56 expression on PCs in BM and on those in peripheral blood. *Conclusions.* In two thirds of MM patients malignant PCs show CD56 expression. Intensity of CD56 expression on PCs varies among particular CD56 positive MM patients. There is relationship between proportion of BM CD56 positive PCs and density (ABC) and intensity (RFI) of expression of this molecule. In half of PCL cases leukemic PCs show CD56 expression.

Myeloma and other monoclonal gammopathies II

0239

FLUORODEOXYGLUCOSE POSITRON EMISSION TOMOGRAPHY IN MULTIPLE MYELOMA, SOLITARY PLASMOCYTOMA AND MONOCLONAL GAMMAPATHY OF UNKNOWN SIGNIFICANCE

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The aim of our study was to evaluate the role of fluorine-18 fluorodeoxyglucose positron emission tomography (FDG-PET) in plasma cell malignancies. A total of 49 pacients were enrolled including 13 patients with newly diagnosed multiple myeloma (MM) and negative bone radiographs, four patients with solitary plasmocytoma, 26 patients with MM in remission but with suspected relapse, and six patients with monoclonal gammopathy of unknown significance (MGUS) with suspected progression to MM or with suspected other malignancy. FDG-PET results were verified by conventional imaging methods, including plain radiographs, magnetic resonance imaging (MRI) and computer tomography (CT). Focally increased FDG uptake was observed in three (23%) of 11 newly diagnosed myeloma patients with negative bone radiographs. The findings were all confirmed by CT or MRI. FDG-PET was negative in two patients with newly diagnosed MM, negative bone radiographs, and without focal infiltration on MRI but with anemia, high monoclonal imunoglobulin and high bone marrow infiltration by plasmocytes. In all other cases FDG-PET negativity in asymptomatic myeloma was associated with favorable prognosis; these patients are without progression after the median follow-up of 14 months. Focally increased tracer uptake was found in five of 26 patients with MM in remission. In four cases it was due to MM relapse, in one case due to ovarian carcinoma. Only in one patient FDG-PET failed to recognize extraosseal progression. Of the 20 patients who had negative FDG-PET scans, only one relapsed 12 months after FDG-PET examination; the remaining 19 patients are without progression with the median follow-up of 15 months. FDG-PET was positive in two of six patients with MGUS. In one case a thyroid carcinoma was later detected, in the other an intestinal tumor was found. We conclude that FDG PET might contribute to initial staging of MM patients with negative bone radiographs and is useful for the follow-up of patients in remission especially in non-secretory MM and in patients with large plasmocytoma (>5 cm) after radiochemotherapy.

0240

TREATMENT WITH BENDAMUSTINE, THALIDOMIDE AND PREDNISOLONE IN ADVANCED MYELOMA PATIENTS: RESULTS OF A PHASE I CLINICAL TRIAL.

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Thalidomide is an active single agent in advanced relapsed or refractory multiple myeloma (MM). Combination of low dose thalidomide with bendamustine and prednisolone might be a way to maintain efficacy of the drug without dose limiting toxicity (DLT). The treatment consists of a fixed dose of bendamustine (60 mg/qm) day 1, 8, and 15 and prednisolone (100 mg) day 1, 8, 15, and 22. At the same time, thalidomide was given in patients cohorts with escalating doses, starting with 50 mg to a maximum of 200 mg daily. 8 patients (4 after conventional chemotherapy and 4 after APBSCT) were enrolled at each dose level. Cycles were repeated every 28 days for a minimum of 2 and a maximum of 10 cycles until a maximal response was achieved, a DLT or a disease progression were observed. 23 patients (8 in the first dose level with 50 mg thalidomide, 8 in the second dose level with 100 mg and 7 patients in the third dose level with 200 mg) are enrolled until now. The number of prior treatment regimens was 2 or more in all patients. 6 patients were refractory for the last treatment. Median age was 67 years (range: 40-78). All patients completed 2 cycles of BPT-treatment and were hence evaluable. Response was assessed using EBMT criteria modified to include near complete remission (nCR) and very good partial remission (VGPR). 21 of 23 patients responded after at least 2 cycles of chemotherapy with 3 CR, 5 VGPR, 11 PR and 2 MR. 2 patients had stable disease. With a median follow up of twelfe months, EFS and OS at twelfe months were 47% and 87%, respectively. Most common site

effects were constipation (10 patients WHO grade 1, 8 patients WHO grade 2), polyneuropathy (14 patients WHO grade 1, 2 patients WHO grade 2) and somnolence (4 patients WHO grade 1). None of the 23 patients developed dose-limiting hematoxicity as defined by an ANC < 1,0 Gpt/L for > 7 days or an ANC < 0,5 Gpt/L for > 3 days or platelet count < 25 Gpt/L. Short neutropenia was reported in 8 patients (WHO grade 3 and 4) but no thrombocytopenia was observed. BPT with a dose between 50 and 200 mg thalidomide daily is well tolerated in patients with relapsed or refractory MM.

0241

CANTHARIDIN, A DERIVATIVE OFBLISTER BEATLES INDUCES APOPTOSIS IN MULTIPLE MYELOMA CELLS VIA INHIBITION OF IL-6-INDUCIBLE STAT3 PATHWAY: NEW AGENT FOR SIGNAL TRANSDUCTION THERAPY OF MULTIPLE MYELOMA

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Background. Multiple myeloma remains incurable despite the use of high-dose chemotherapy with hematopoietic stem cell transplantation; therefore, novel therapeutic approaches are urgently needed in clinical settings. The understanding that has recently been gained into the biology of myeloma has led to the development of biological treatments, which target the myeloma cells and its microenvironment. These agents have shown remarkable activity against refractory myeloma in early clinical trials, but prolonged drug exposure may result in the development of *de novo* drug resistance. Therefore, the identification and validation of novel targeted therapies to overcome drug resistance and improve patient outcome are necessary. *Aims.* Previous reports suggest that IL-6 promotes survival and proliferation of myeloma cells through the phosphorylation of STAT3. Thus, compounds that suppress STAT3 phosphorylation have the potential for the treatment of myeloma. Recent studies have shown that Chinese traditional medicine cantharidin (CTD), a derivative of Blister Beatles, induces apoptosis in hepatoma, colon cancer, and leukemia cells. Therefore, we assume that CTD has a potency to induce apoptosis in myeloma cells, and may lead to a novel targeted therapeutic approach. *Methods.* To address our hypothesis, myeloma cells (U266, RPMI8226), and fresh myeloma samples from patients were treated with CTD. The effects of CTD on cell growth, apoptosis, cell cycle status, and the signaling pathway were studied. *Results.* CTD inhibited cellular growth of myeloma cells as well as freshly isolated myeloma cells from 5 patients in dose (0-10 μM)- and time (0-48h)-dependent manners with IC 50 of 4.3 μM. Cultivation with 5 μM CTD did not induce cell cycle arrest, but induced apoptosis of myeloma cells and primary cells from patients, but not bone marrow cells from healthy volunteers 24h after treatment. These results suggest that CTD-induced apoptosis is cell cycle-independent manner. Treatment with CTD induced caspase-3 activity in myeloma cells, and it was completely blocked by the pre-treatment with Z-VAD. To address the molecular mechanism of CTD-induced apoptosis in myeloma cells, we next examined the effect of CTD on IL-6 signaling pathway. CTD inhibited IL-6-induced gp130 activation in a time-dependent manner. STAT3 is a transducer of the IL-6 signaling pathway; thus we examined whether CTD could inhibit the STAT pathway. CTD inhibited phosphorylation of STAT3 at tyrosine 705 residues as early as 30 min after treatment, and down-regulated the expression of anti-apoptotic Bcl-xL. It has reported that STAT3 directly binds and activates the transcription of Bcl-xL gene promoter, resulting in the induction of the expression of Bcl-xL in IL-6-treated myeloma cells. Our results suggest that the inactivation of STAT3 and the down-regulation of Bcl-xL may contribute to CTD-induced apoptosis in myeloma cells. *Conclusions.* In conclusion, we report here for the first time that CTD induces apoptosis in various myeloma cells and primary myeloma cells in cell cycle-independent manner. Down-regulation of Bcl-xL with modulation of STAT3 in IL-6-mediated signaling pathway plays an important role in CTD-induced apoptosis in myeloma cells. Therefore, CTD is one of the promising candidates for the new therapeutic agent as a signal transduction therapy of myeloma.

0242

CITRULLINE CONCENTRATION AFTER HIGH-DOSE MELPHALAN IN AUTOLOGOUS HSCT RECIPIENTS

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Background. Mucosal damage to the intestines induced by intensive myeloablative conditioning for an allogeneic HSCT can be determined by the concentration of citrulline which is a functional marker of small intestinal enterocytes. However, there are no data available about the kinetics of citrulline levels after high-dose melphalan used to prepare for an autologous HSCT. Aims We were interested to know whether and when the citrulline concentrations declined after starting myeloablative therapy. Methods We selected 29 patients who underwent an autologous HSCT following conditioning with HDM 100 mg/m^2 HSCT day -3 & -2. We collected plasma samples from each patient via a central venous catheter at 9.00 hour on the first day of conditioning therapy and 3 times per week (Monday,Wednesday, Friday) thereafter until discharge. The samples were stored frozen until citrulline concentrations could be determined by HPLC.[1] Oral mucositis was registered using a Daily Mucositis Score. Results The baseline mean citrulline concentration was 28 mM which is lower than the 35 mM that is found normally. The mean citrulline concentrations declined rapidly thereafter reaching a nadir of 6.7 μmol/L 11 days after starting HDM which is HSCT day +7. Citrulline concentrations then only increased gradually and were still significantly low at 12 mM when patients were discharged. The most severe oral mucositis coincided with the nadir of citrulline. Conclusion Citrulline appears a valuable marker of small intestinal mucosal barrier injury induced by HDM to prepare for an autologous HSCT.

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0243

PLASMA CELL PROPIDIUM IODIDE (PC-PI/CD138) AND ANNEXIN V (PC-AI/CD138) INDICES IN MULTIPLE MYELOMA AND MONOCLONAL GAMMOPATHY OF UNDETERMINED SIGNIFICANCE EARLY PREDICTORS OF TRANSFORMATION?

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Background. Propidium iodide and annexin V indices have a close relation to prognosis in multiple myeloma (MM) and have been proved to be significant and independent prognostic factors in its evaluation. The aim of this study is to evaluate these indices also in monoclonal gammopathy of undetermined significance (MGUS) in correlation with MM, especially with stage I (D-S) to determine their importance as early predictors of transformation from benign into symptomatic phase of the disease. *Methods.* Analysed group consists of 257 patients (70 MGUS and 187 MM patients -21 st. I, 78 st. II and 88 st. III), all at the time of diagnosis, before therapy. 20 of MGUS patients were measured also in regular 6-12 month intervals to evaluate the course of MGUS. Proliferative activity of plasma cells was measured using propidium iodide index (PC-PI /CD138), rate of apoptosis using annexin V index (PC-AI/CD138), followed by method of flow-cytometry (DNA- Prep Reagents Kit, Coulter, Software Multicycle fy. Phoenix). For statistical estimation Student´s t-test, ANOVA and non-parametric Mann-Whitney test were used. *Results.* Within the evaluation of propidium iodide and annexin V in MGUS and MM there was a significant difference between the values of both indices ' in MGUS the values of proliferation were lower (M: 1,9%) than in MM (M: 2,6%, p<0,0001) and the values of apoptosis were higher (M: 7,45% vs 4,6%, p<0,0001). If we depicted only stage I MM there was also statistically significant difference in proliferation when comparing with MGUS (M: 1,9% vs 2,3%, p=0,016). In apoptosis we found higher values in MGUS (M: 7,45% vs 6,2%), however not statistically significant (p=0,121). In next step we tried to analyse differences in PC-PI and PC-AI within the stages of MM. The corresponding medians of PC-PI were for stage I, II and III (D-S) values 2,3%, 2,6% and 2,75%, none of them being significant (p=0,539). For apoptosis the

results were similar - for stage I, II and III values 6,2%, 4,9% and 4,3%, p=0,196. Finally we compared the values of PC-PI and PC-AI within the course of 20 MGUS patients - there was no statistical significance between the values, either. *Conclusion.* Our measurements support the hypothesis of PC-PI and PC-AI being independent prognostic factors and also the indicators of early transformation of MGUS into MM. Within the course of MGUS there exists no significant change in either of the indices, however, when transforming into symptomatic multiple myeloma, there is a significant increase in PC-PI together with decrease of PC-AI. The above results also confirm the major importance of proliferation in the process of transformation into MM - there is significant difference even between MGUS and stage I MM, on the other hand, decrease in apoptosis is probably not the fundamental part of this process. Measurement of proliferation and apoptosis contributes to the assessment of MM prognosis, and plays also a prominent role in the evaluation of the course of MGUS, especially as an early predictor of transformation into multiple myeloma.

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0244

EPIDEMIOLOGY OF ANEMIA IN 720 PATIENTS WITH MULTIPLE MYELOMA: RESULTS FROM EUROPEAN ANAEMIA SURVEY

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Background. Although anemia is a common complication of multiple myeloma (MM) patients (pts), information on the evolution of anemia during follow up, relation with age and performance status, risk factors for anemia and treatment practices was not available. *Aims.* Identify the incidence, prevalence and evolution of anemia during an up to 6 months follow up period, analyse possible correlations between anemia and clinical characteristics, identify risk factors for evolution of anemia (in pts with myeloma and lymphoma) and study patterns of anemia treatment in European myeloma pts. *Methods.* 720 patients with multiple myeloma (male 52% and female 48%) were enrolled into a prospective, epidemiologic survey, ECAS (European Cancer Anemia Survey), which included an additional 1640 pts with lymphoma (L) and a total of 15,370 pts with cancer at any stage of their disease. Survey data were collected for up to 6 data points or 6 months of scheduled visits (Ludwig, EJC 2004; 40 (15): 2293-2307). *Results.* Median age in MM pts was 65.7 years (range 31-94), with 28% of patients presenting with age <60 years (yrs), 32% with age 60-69 yrs, and 40% with age ≥70 yrs. 28% of the 720 pts with MM were newly diagnosed; 55% had persistent/recurrent disease and 17% were in remission. In terms of cancer treatment, 50% were receiving chemotherapy (CT), 46% were not receiving any cancer treatment, with the remainder receiving radiotherapy or concomitant CT and radiotherapy. At enrollment, 69% of patients were anemic (Hb <12g/dL), 30% had Hb < 10 g/dL and 39% Hb of 10 to 12 g/dL. 85% were anemic at some time during the survey. 78% of those <60 yrs, 85% of those 60-69 yrs and 90% of those 70+ were ever anemic. 44% had a WHO score of 2-4. The incidence of anemia in MM who were not anemic at enrolment and who started CT during ECAS was 75%. Incidence of anemia increased with increasing age (60% in pts < 60 yrs, 88% in those 60-69 yrs and 100% in those 70+). Adverse WHO score correlated with low Hb (r=-0.346). Despite the 59% of those who became anemic having a nadir Hb < 10 g/dL, 41% received no anemia treatment, 2% received iron, 22% transfusion and 35% received epoetin. Logistic regression analysis of MM/L pts revealed 4 variables significantly predicting anemia development: Initial Hb (adjusted odds ratio (AOR) 4.2), persistent/recurrent disease (AOR 1.5), female gender (AOR 2.8), and treatment with platinum-based CT (AOR 5.5) were found to independently predict anemia (p<0.001). *Conclusions.* Frequency of anemia in MM pts remains substantial and important: prevalence of anemia (ever anemic) was high (85%) in MM pts, increased with age and correlated with poor PS. Follow up during the 6 month post-enrollment period indicated that 75% of initially non-anemic pts developed anemia after starting CT. Anemia treatment was given to 41% of ever anemic MM pts, although 59% had at least once Hb levels <10g/dL. With the identification of important risk factors, anemia management in MM pts could be improved.

0245

A PHASE I/II STUDY OF ARSENIC TRIOXIDE, BORTEZOMIB, AND ASCORBIC ACID IN RELAPSED OR REFRACTORY MULTIPLE MYELOMA

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Background. Arsenic trioxide (ATO), a trivalent arsenite salt, is believed to exert its cytotoxic effect by causing DNA fragmentation characteristic of apoptosis. Clinical studies have shown that ATO has antitumor activity as a single agent in patients with relapsed or refractory multiple myeloma (MM). Bortezomib (B) is a proteasome inhibitor that is currently approved for the treatment of relapsed or refractory MM. Preclinical studies have shown that combining ATO and B results in synergistic antitumor activity against human MM cells in tissue culture and xenograft animal models. Furthermore, the addition of ascorbic acid (AA) can sensitize human MM cells to the cytotoxic effects of ATO. These observations suggest that the combination of ATO/B/AA may be an effective treatment regimen for patients with MM. *Aims.* The primary aim of this study was to determine the safety and tolerability of the ATO/B/AA regimen in patients with relapsed or refractory MM. The secondary aims were to determine overall response rate, time to response, time to progression, progression-free survival, and overall survival in these patients. *Methods.* Patients with relapsed or refractory MM were enrolled in this Phase I/II dose-escalation trial in 6 cohorts. Patients were given ATO (0.125 or 0.250 mg/kg), B (0.7, 1.0, or 1.3 mg/m^2), and a fixed dose of AA (1000 mg) IV on days 1, 4, 8, and 11 of a 21-day cycle for a maximum of 8 cycles. *Results.* At the time of this interim analysis, 22 patients (median age, 63 years) have been enrolled, and accrual has been completed on all cohorts. This group had failed a median of 4 (range, 3-9) prior therapies. One occurrence of grade 4 thrombocytopenia was observed. One occurrence of asymptomatic arrhythmia led to patient withdrawal. All other adverse events were grade 1 or 2. For the 21 patients evaluable for efficacy, objective responses were observed in 9 patients (43%), including 2 complete (CR; 10%), 2 partial (PR; 10%), and 5 minor (MR; 24%) responses. Only 1 (1 MR) of 6 patients receiving the lowest dose of B (0.7 mg/m^2) showed a response, whereas 4 (1 CR and 3 MR) of 6 patients receiving the middle dose of B (1.0 mg/m^2) responded, and 4 (1 CR, 2 PR, and 1 MR) of 9 patients receiving the highest dose of B (1.3 mg/m^2) responded. *Conclusions.* The ATO/B/AA regimen was well tolerated by the majority of patients and produced objective responses in 43% of the patients in this heavily pretreated group. Eight of 15 patients receiving the higher doses of B had clinical responses to this regimen. The results of this Phase I/II study warrant further clinical evaluation of the ATO/B/AA combination regimen for the treatment of patients with relapsed or refractory MM.

0246

PREVALENCE OF RAS GENE MUTATIONS IN THE CONTEXT OF A MOLECULAR CLASSIFICATION OF MULTIPLE MYELOMA

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Background. Earlier studies have reported that activating mutations involving *RA* genes, in particular *NRAS* and *KRAS*, occur frequently in multiple myeloma (MM). The reported prevalence of mutated tumors varies from 10 to 40% at presentation, rising to 70% at relapse, suggesting a role of this lesion in tumor progression. Notably, the occurrence of such mutation in MGUS and indolent tumors is very low. Mutations of *KRAS*, but not *NRAS*, have been found to be associated with higher bone marrow burden and shorter survival. *Aims.* In the present study we investigated the prevalence and type of *RAS* mutations in MM in the context of a proposed molecular stratification, named as *TC classification*, based on the presence of IGH translocation and dysregulation of cyclin D genes in MM. *Methods.* The presence of *NRAS* and *KRAS* gene mutations was investigated in a panel of 82 MM at diagnosis, 13 patients with extramedullary myeloma or plasma cell leukemia, 9 patients with

MGUS and 4 normal controls. The mutation analysis was performed by RT-PCR and direct DNA sequencing on purified CD138+ plasma cell populations (>90%). The expression levels of the three cyclin D genes in MM patients were derived from the gene expression profiling (GEP) data generated using high-density oligonucleotide arrays. GEP data were analyzed using unsupervised (two-dimensional hierarchical clustering) and supervised (SAM, Significant Analysis of Microarrays) approaches. *Results.* Mutations were found in 16/82 (20%) myeloma patients, in 2/13 (20%) PCL samples and in none of the MGUS patients. In 11 MM patients the mutation involved the *NRAS* gene at codon 13 (3 patients) and 61 (8 patients), and the KRAS gene at codon 12 (4 patients) and 61 (1 patient), respectively. PCL patients were both harboring a NRAS mutation at codon 61. Mutations were found in patients included in all TC groups: 4 patients in TC1 (23.5%), 5 in TC2 (28%), 3 in TC3 (11.5%) and 2 patients in both TC4 (12.5%) and TC5 (50%) groups. Although the higher frequency of mutations observed in TC1 and TC2 groups, this finding did not reach a significant statistical level. No significant correlation was found with chromosome 13q deletion, trisomy of chromosome 11, or 1q amplification. Unsupervised analysis of gene expression profiles of the 82 patients did not show any particular evidence of clustering of tumors with *RAS* mutations. A supervised analysis approach, comparing the *RAS* mutated MM (16) cases versus wild-type (66) tumors in the complete dataset as well as in the TC1, 2, 3 or 4 groups, did not allow the identification of differentially expressed transcripts. *Conclusions.* Our study confirms the previous evidences reported by us and others and indicates that *RAS* mutations did not correlate at significant levels with specific genetic lesion or molecular features in MM.

0247

PERSONAL HISTORY OF REPEATED PNEUMONIA IS ASSOCIATED WITH INCREASED RISK OF MULTIPLE MYELOMA

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Background. In Europe and the U.S. a total of more than 35,000 multiple myeloma (MM) cases are diagnosed annually. Although the etiology of MM remains unclear, associations between MM and past history of disorders characterized by chronic immune dysfunction such as pneumonia have been observed in limited clinical and epidemiological studies. *Aims.* To evaluate risk of MM associated with a personal history of airway infections. *Methods.* Using population-based linked registry data from Denmark, we conducted a case-control study including 4,476 MM cases diagnosed 1977-1997 and 16,727 age and gender matched controls. All individuals were linked with the Danish Inpatient (1977-1997) and Outpatient (1994-1997) Register to gather information on discharges listing any of the following coded airway infections: tuberculosis, pneumonia, bronchitis, unspecified lower airway infection, laryngitis, nasopharyngitis/pharyngitis, unspecified upper airway infection, sinusitis, otitis media, and influenza. We calculated odds ratios (ORs) and 95% confidence intervals (CIs) as measures of relative risks for each condition using logistic regression. Airway infection data were restricted to those that occurred more than one year before MM diagnosis for cases and their corresponding controls. In models including multiple prior airway infections, we examined the association between MM risk and number of events (1, 2, and 3+) and time from discharge listing a defined airway infection until MM diagnosis (1-5, 5-10, and 10+ years latency). Observed associations were stratified by age at MM diagnosis (<65 vs. ≥65 years). *Results.* We found significantly increased risk of MM associated with personal history of pneumonia (OR=1.7, 95%CI 1.5-2.1; 207 cases, 437 controls). In analyses stratified by latency, significant 2.1-fold (95%CI 1.7-2.6; 149 cases, 271 controls) elevated MM risk was observed in the 1-5 year latency interval but not in the 5-10 year (OR=1.2, 95%CI 0.9-1.7; 49 cases, 132 controls) or the 10+ year (OR=0.8, 95%CI 0.5-1.4; 18 cases, 62 controls) latency intervals. Among persons with 1 or 2 prior pneumonia events, we found significant 1.6-fold (95%CI 1.3-1.9; 166 cases, 361 controls) and 1.7-fold (95%CI 1.0-2.6, p=0.031; 27 cases, 55 controls) elevated MM risks, respectively. We observed that individuals with 3+ previous pneumonia events had further elevated (OR=2.2, 95%CI 1.1-4.3; 14 cases, 21 controls) MM risk, which was particularly high among older (≥65 years) individuals (OR=2.8, 95%CI 1.2-6.4). Older subjects with 3+ pneumonia events in the 1-5 years latency interval had the most prominent MM risk (OR=3.6, 95%CI 1.2-11.3; 6 cases, 6 controls). We found no association between personal history of other airway infections and MM risk. *Summary/Conclusions.* Personal history of pneumonia was a predictor for risk of MM; particularly among individuals ≥65 years with multiple prior pneumonia events. The increased MM risk subsequent to pneumonia was confined to the 1-5 year latency interval suggesting that pneumonia might be a potential late trigger for MM development, rather than a risk-factor for the precursor of MM, monoclonal gammopathy of undetermined significance (MGUS). Alternatively, pneumonia could be a manifestation of immune disturbances in late-stage MGUS. Future studies examining underlying mechanisms of the observed findings may provide insights to the etiology of MM.

0248

MINIMAL RESIDUAL DISEASE CAN BE DETECTED IN ALMOST ALL MULTIPLE MYELOMA PATIENTS IN REMISSION USING A COMBINED APPROACH OF FIVE-COLOUR FLOW CYTOMETRY AND INTERPHASE FISH ON SUBSEQUENTLY SORTED PLASMA CELLS

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Background. Translocations involving IGH, del(13q) and del(17p) are frequently found in multiple myeloma (MM), smoldering myeloma (SM) and monoclonal gammopathy of undetermined significance (MGUS) by fluorescence *in situ* hybridisation (FISH) and are shown to be of prognostic relevance. However, adequate FISH detection may be hampered by low-level bone marrow infiltration, in SM and MGUS and also in MM, at diagnosis and especially during therapy. Plasma cell (PC) targeting strategies are thus mandatory to increase sensitivity of the FISH assay. *Aim.*We aimed at demonstrating the feasibility and high sensitivity of a combined approach of five-colour flow cytometry (FCM) and interphase FISH on subsequently sorted PC. Therefore, using this approach, we have analysed bone marrow samples from patients at different stages of disease and therapy, all containing low percentages of PC. *Methods.* A total of 32 bone marrow aspirates were analysed, including samples from patients with MGUS (n=12), SM (n=3), MM at diagnosis or relapse (n=6) and MM in partial or complete remission (CR) (n=11). Cytological analysis of bone marrow smears showed PC percentages in the range 0.5% to 11%. Expression of molecules of interest in PC disorders (CD138, CD38, CD56, cyIg light chain (L)) was routinely investigated using five-colour FCM on a FACS-Aria (BD, US). CD56±IgL cell populations were subsequently sorted and spotted on slides with the same instrument. The high purity of the sorted cells (mean 95%) was demonstrated by reanalysing the cells by FCM and by microscopy. Dual colour interphase FISH with probes for IGH, 13q, 17p and 12 (control) (Vysis, Abbott, US) was applied on the sorted PC as well as on the corresponding bone marrow smears. *Results.* Five-colour FCM showed the presence of an aberrant PC population, by the presence of IgL restriction and/or the expression of CD56 in 27 of 32 samples. On sorted PC, 24 (MM, SMM, MGUS) out of 32 samples (75%), including 4 FCM negative samples, displayed at least one abnormality with FISH (del(13q), IGH translocation and del(17p) in respectively 15, 11 and 6 of 24 cases), versus only 8 on the corresponding smears (25%). Of 11 samples derived from patients in partial or complete remission, all except one showed aberrant PC by five-colour FCM and/or by FISH on sorted PC, indicating the presence of minimal residual disease (MRD). *Conclusion.* FISH on flow sorted PC improves the detection of chromosomal aberrations in plasma cell disorders and is very well applicable in the routine diagnostics of MGUS, SM and MM for prognostic evaluation. Importantly, using the combined approach of five-colour FCM and interphase FISH on subsequently purified plasma cells, we were able to detect MRD in almost all of MM patients in remission. We propose this approach as a valuable alternative for other MRD detection methods such as flow cytometry alone and allele-specific PCR.

0249

BORTEZOMIB TRANSIENTLY INHIBITS OSTEOCLAST ACTIVITY IN CELL CULTURE CONDITIONS MIMICKING *IN VIVO* INTERMITTENT TREATMENT

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Background. Bone disease induced by multiple myeloma (MM) leads to severe pain, high risk for collapse of vertebral bodies and fractures of the major weight-bearing bones. It is due to acute degradation of bone matrix by osteoclasts, not coupled with new bone formation by osteoblasts. MM-induced bone disease is currently treated with bisphosphonates, highly effective bone resorption inhibitors, which do not stimulate but rather inhibit bone formation. Furthermore bisphosphonates may cause renal damage and osteonecrosis of the jaw. Therefore,

it is important to reconsider the management of MM bone disease in long-term treatment. Recently, preclinical studies have reported that the proteasome inhibitor Bortezomib used for the treatment of MM patients can stimulate bone formation, and that in MM patients treated with Bortezomib serum levels of bone formation markers are increased. *Aims.* In this study, we have investigated whether Bortezomib may inhibit osteoclast activity. *Methods.* Osteoclasts were differentiated from pure populations of blood derived CD14-positive monocytes cultured with M-CSF and RANKL for 6-7 days. Cells were treated with Bortezomib at different concentrations in a continuous mode. It has been reported that prolonged inhibition of proteasome activity may be toxic for any cell type and *in vivo* pharmacodynamic studies have shown Bortezomib to be eliminated from the vascular compartment as soon as 30min after intravenous injection, displaying maximal inhibitory activity of the proteasome within 24 hours subsiding rapidly thereafter. Therefore, bortezomib was also given intermittently to mimick the *in vivo* situation. Osteoclast differentiation and activity were assessed by measuring Tartrate-Resistant Acid Phosphatase (TRAcP) activity in the medium. Cell viability was determined with Celltiter Blue measuring metabolic activity. To extend our observations to the clinical situation, serum levels of CTX-I, a bone resorption marker, were measured during the 3 days following therapeutic Bortezomib administration in a single patient. *Results.* Continuous treatment with Bortezomib at 4nM and higher concentrations proved to be highly toxic for differentiating osteoclasts (cultures in presence of M-CSF+RANKL) but also monocytes (cultures in presence of M-CSF only) during a 7-day culture. However, a 6-hour-pulse treatment with Bortezomib every third day, was not toxic to primary monocytes, even at a concentration as high as 25nM and a culture period as long as 7 days. In this condition, TRACP activity of osteoclasts was strongly inhibited by Bortezomib within the first 24 hours post-pulse (65% inhibition at 25nM Bortezomib) but the activity returned to the control level after 72 hours. In the patient serum serum levels of CTX-I decreased during the first 48 hours after each Bortezomib injection (n = 3), and tended to increase again after 72 hours suggesting a partial recovery of osteoclast activity between each dose. *Conclusions.* Our results suggest that Bortezomib temporarily inhibits osteoclast activity *in vitro* and *in vivo*. This transient inhibition of osteoclasts could be an advantage compared to the more persistent inhibition of osteoclast activity by bisphosphonate since recent reports suggested that formation of new bone requires at least a transient activity of osteoclasts. Further clinical studies are warranted to validate our findings.

0250

COMBINATION THERAPY EFFECTS OF LENALIDOMIDE IN FGFR3 MULTIPLE MYELOMA CELL LINES

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Background. Lenalidomide (Revlimid®) has recently been approved for the treatment of a subset of myelodysplastic syndromes, and is being evaluated as treatment for a broad range of other hematology and oncology conditions, including multiple myeloma (MM), chronic lymphocytic leukemia and solid tumor cancers. Data from Phase II and Phase III studies of lenalidomide plus dexamethasone in MM patients show significant benefits of combination therapy versus dexamethasone alone, in terms of median time to disease progression and overall survival. A subset of MM patients (10-20%) are known to have a t(4;14) translocation that results in the overexpression of oncogenes FGFR3 and MMSET/WHSC1. FGFR3 is a tyrosine receptor kinase that is activated by the pro-angiogenic growth factors aFGF and bFGF. FGFR3 is not normally expressed in B cells but is overexpressed and sometimes has a constitutively activating mutation in multiple myeloma cells with t(4;14). We hypothesized that FGFR3+ cells may have enhanced sensitivity to lenalidomide inhibition. *Aims.* Given the involvement of FGFR signaling in angiogenesis and the anti-angiogenic activity of lenalidomide, we studied the effect of lenalidomide on proliferation signals in FGFR3+ MM cells. *Methods.* Three FGFR3+ (NCI-H929 (t(4;14), wt FGFR3), LP-1 (t(4;14), FGFR3 F384L), and OPM-2 (t(4;14), constitutive FGFR3 K650E)) and six FGFR3- (no t(4;14)) control MM cell lines (JJN3, SK-MM-2, EJM, RPMI-8226, Karpas-620 and KMS-12) were tested. Cells were incubated with compounds for 72 hours and assayed by 3H-thymidine incorporation. IC50s were calculated by nonlinear regression analyses with GraphPad Prism. *Results.* The lenalidomide sensitivity in cell proliferation assays is NCI-H929 > Karpas-620 > LP-1 > SK-MM-2 > EJM, OPM-2 > JJN3 > RPMI-8226 > KMS-12 indicating no correlation between lenalidomide anti-proliferative activity and FGFR3 expression. Thalidomide had little if any effect (IC50>100 mM) in all cells tested. To study the effect of lenalidomide in combination with other chemotherapeutic agents in FGFR3+ MM cells, three FGFR3+ MM cell lines (NCI-H929, LP-1 and OPM-2) were treated with lenalidomide in combination with dexamethasone (Dex), doxorubicin (Dox), and vincristine (Vinc). In FGFR3-negative cells (RPMI-8226), lenalidomide was only partially additive with Dex, and completely non-additive with the other three agents. In the FGFR3+ cells, lenalidomide was partially additive with Dex and Vinc. The len+Vinc combination was also examined in 5 addition FGFR3- multiple myeloma cell lines and partial additivity was observed in 2 out the 5 cell lines tested. In the constitutively active FGFR3+ cells, the len+Dex and len+Vinc combinations were fully additive. The len+Dox combination was partially additive in two out of three FGFR3+ MM cell lines. *Conclusions.* No correlation was found between lenalidomide anti-proliferative activity and FGFR3 expression. In four MM cell lines (RPMI-8226, NCI-H929, LP-1, OPM-2), the len+Dex combination is better than either agent alone, which correlates with the clinical observation that this combination in MM patients is better than Dex alone. Our data suggest that the len+Dex combination may also be beneficial in an FGFR3+ MM population. These data also provide new evidence to suggest that the len+Vinc combination may be better than either agent alone against multiple myeloma clone proliferation.

0251

A NOVEL *IN VIVO* ANIMAL MODEL FOR HUMAN MULTIPLE MYELOMA BASED ON BIOLUMINESCENCE IMAGING OF TUMOR CELL GROWTH

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Preclinical testing of new therapeutical strategies or new cytotoxic drugs for the treatment of multiple myeloma (MM) requires animals models that closely resemble human disease and that allow quantitative evaluation of the applied therapy. Here we present a novel *in vivo* MM model by engraftment with U266 or RPMI-8226/S cells, both of human origin, into RAG2cg double knock-out mice (RAG2GC). These mice are immune deficient because they lack T-, B and NK cells and the mice easily accept human cells (van Rijn *et al.*, Blood 2003, Rozemuller *et al.*, 2004). In this model we introduce the use of luciferase gene marking of the MM cells and applying Bioluminescence imaging (BLI) in living animals for measuring the initial growth of the MM cells and the response to treatment. After intravenous injection of 2×10^6 cells MM cells engraftment and outgrowth occurred in all mice but it was limited to the bone marrow compartment, thus resembling human MM. FACS analysis revealed the presence of human CD45, CD138 and CD38 positive myeloma cells in a variety of examined bone specimen. Infiltration into other organs was not observed. MM cells were transduced with a GFP-Firefly luciferase (fLuc) fusion gene. When luciferase converts the substrate luciferin, photons are emitted that can be registered by using sensitive CCCD cameras. The absolute number of photons that are produced correlates (in our application) with the local tumor mass. Mice were injected i.v. with GFP-fLuc 2×10^6 cells MM cells (U266 or RPMI8226/S) and then imaged weekly using BLI. Within 2 weeks after injection significant BLI signals were detectable. Per mouse 5-10 foci showed luciferase activity, predominantly in the pelvic region, skull, limbs and the spine. All mice were examined weekly with BLI. We observed that the amount of light produced at the various foci of tumor growth, within an individual mouse as well as in between mice, showed a comparable increase. After 9-12 weeks all mice were killed due to excessive tumor growth. Growth curves that were made on the basis of subsequent BLI images revealed exponential growth of the total tumor mass per mouse as well as for the individual foci of MM growth in each mouse. All curves show similar growth kinetics with an average population doubling time of approximately 5-6 days. The range in which tumor growth can be monitored with BLI (and as a consequence also the response to treatment) spans 3-4 decades. The BLI signals could postmortem be confirmed by flow cytometry of GFP+ cells in affected bones. The major advantage of this model is the option for quantitative evaluation of the effect that a given treatment has on the tumor load. In conclusion, we have developed a novel *in vivo* model to study the characteristics of homing and outgrowth of MM and we show that it can be used for quantitative evaluation of the efficacy of the therapeutic intervention.

0252

RELATIVE QUANTIFICATION OF TUMOR ASSOCIATED ANTIGENS MAGE-A1 AND MAGE-A3 IN MULTIPLE MYELOMA

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Background. Multiple myeloma (MM) is a malignant plasma cell neoplasm that often is preceded by a common pre-malignant monoclonal expansion of plasma cells called monoclonal gammopathy of undetermined significance (MGUS). MGUS is reported to be present in 1% of the adult population and to progres to MM at a rate of 1% per year. MM is an incurable tumor characterized by clonal expansion of malignant plasma cells in the bone marrow. The MAGE genes encode antigenic peptides that are presentd by HLA class I molecules and that are recognized on human tumors by T lymphocytes. They are activated in a variety of malignant neoplasma while remaining silent in normal tissues with the exception of testis and ocassionally placenta. Presence of RNA transcripts encoding members of the MAGE gene family in myeloma tumor cells and cell lines has been documented. *Aims.* The aim of this study is to evaluate the possibility of using these genes as molecular markers of the progression MGUS to multiple myeloma and the early relapse of the MM. This abstract covers our pilot and preliminary *Results. Methods.* Total of 50 samples from bone marrow were evaluated: 25 samples from myeloma patients, 8 samples of patients with early stage of MM who did not required treatment (smoldering MM 2x and stage IA 6x), 5 samples of MGUS patients, 9 samples of normal healthy donors served as control group. Total RNA was evaluated by RT-PCR and then by real-time PCR using FRET probes on the LightCycler instrument (Roche). For relative quantification we used G6PDH housekeeping gene as external standard. As positive control we used myeloma cell line U266. *Results.* None from samples of 9 healthy donors did show expression of MAGE. Only 1 of 5 (20%) samples from MGUS patient showed expression of MAGE-A1. Five (62,5%) from 8 patients with early stage of MM (IA and smoldering) showed expression of MAGE. On the contrary 11 (44%) of 25 samples from MM patients showed expression of at least one gene MAGE-A1 or MAGE-A3 or both (7 cases). *Summary/ Conclusions.* We have confirmed that expression of MAGE is not present in samples of healthy donors. There is an obvious correlation between expression of the MAGE genes and early-late stage of the disease as our preliminary evalaution confirmed the detection of low expression levels of MAGE-type mRNA in bone marrow from patients with MGUS and early stage of MM. It is possible that MAGE antigen monitoring may predict the evolution towards more advanced disease as well as this metod should be used for monitoring minimal residual disease in patients with MM. The prospective evaluation is under way. The actual results covering total of 15- 50 evaluated patients in conclusive groups will be presented. This work is supported by grant of the Ministry of Education, Czech Republic, LC06027.

0253

POST RELAPSE AND OVERALL SURVIVAL OF PATIENTS WITH MULTIPLE MYELOMA THAT PROGRESSED AFTER DECEMBER 1998

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Background. The prognosis of multiple myeloma (MM) patients progressing after autologous BMT (ABMT) was documented to be poor, ranging between 14-18 months in various reports. Since December 1998, a variety of novel methods were introduced for the salvage therapy of MM, namely Thalidomide, reduced intensity allogeneic stem cell transplantation (RISCT) and later on Bortezomibe and Lenalidomide. *Aims.* To evaluate the impact of the introduction of novel methods in progressing MM. *Methods.* We report the outcome of a non-selected group of MM patients that progressed from ABMT after December 1998 and were treated in our center. The treatment strategy for this group of patient was based on the nature and the risk score of the relapse, according to the following mile stones: 1. Treatment only at clinical indication; 2. Thalidomide with or without steroids: A. As first line of salvage therapy. B. At relapse from RISCT in a combination with donor lymphocyte infusion (DLI); 3. RISCT or ABMT: A. for consolidation of response in patients resistant to thalidomide (after an induction of response with platinum containing regimen) and /or with high risk responding relapse. B. At escape from thalidomide effect; 4. Bortezomib and Lenailidomide: A. in patients resistant to or escaping from Thalidomide effect, with an attempt to consolidate response by high dose therapy with allogeneic or autologous stem cell support. B. At progression from RISCT that did not respond to DLI and Thalidomide. *Results.* 84 patients (pt's) that their disease progressed after ABMT between December 1998 and May 2004 were enrolled. All patients were treated with Thalidomide as first salvage therapy at a clinical indication, followed by the various options according to the scheme. At a later stage, 32 patients underwent RISCT (22 from related and 10 from unrelated donors) and 13 patients had an ABMT. 16 patients were treated with Bortezomibe and 8 patients received Lenalidomide, for further progression. The median interval from detection of progression to initiation of therapy was 5.5 months. Response rate to thalidomide + steroids was 59% with a median duration of response (for responders not transplanted immediately at response) being 15 months (the longest exceeding 5.5 years). Transplant related mortality in RISCT was 22%. The 3 years overall survival (OS) for all the patients that underwent RISCT is 42%, and for those transplanted at response 61%. The median OS rate from progression, of the entire group of 84 patients, is 39 months. The median OS from first ABMT of this group is 84 m. *Summary*. The introduction, since 1998, of novel tools for the treatment of progressing MM, significantly prolongs the post relapse and the overall survival of patients with MM that undergo ABMT as a part of the initial therapy.

0254

OPG/ RANKL SYSTEM IN MULTIPLE MYELOMA

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Background. According to the contemporary 'convergent' hypothesis the major osteoresorbtive and antiresorbtive factors converge to the system osteoprotegerin (OPG)/receptor activator of nuclear factor kB-ligand (RANKL) and influencing its delicate balance they affect osteoclast proliferation, activation and apoptosis. Clinical results concerning the importance of the system in myeloma bone disease (MBD) are controversial. *Aims.* To analyse the serum levels of OPG and RANKL in patients with multiple myeloma (MM) and their correlations with clinical stage (Durie et Salmon), degree of MBD (according to the Merlini scale) and basic parameters of disease activity. *Methods.* We studied 66 newly diagnosed patients with MM, 29 male, 37 female, median age±SD: 61,8±8,6; range: 45-81years. In I / II / III clinical stage were 13,7% / 33,3% / 53,0% patients, renal failure (RF) was found in 40,9%, MBD in 84,8%, hypercalciemia in 31,8%. Serum levels of OPG and RANKL (ELISA kits Biomedica, Vienna) were compared to a control group of healthy individuals (n=30). Statistics were performed by SPSS for Windows v. 11.0. *Results.* OPG levels were higher in myeloma patients: 5,36±0,46 pmol/l vs 3,77±0,33 pmol/L (p<0,001) but OPG/creatinin ratio (thus eliminating the influence of RF) does not differ between the groups. The lowest OPG levels were measured in patients with MBD grade 2+3: 4,26±0,36. A positive correlation between β2-microglobulin and OPG was found- p<0,001; r= +0,375. We found strong negative correlations of OPG/creatinin with clinical stage (p<0,001; r= -0,616), MBD (p<0,001; r = -0,521) and bone marrow plasmocytic infiltration (p<0,001; r= -0,530). Levels of RANKL were higher in MM compared to controls: 0,458±0,046 pmol/L vs 0,203±0,031 pmol/l (p<0,001).

Table 1. Rankl and Rankl/OPG tatio-clinical correlations.

Parameter	*Rankl*		*Rankl/Opg*	
	p	*r*	*p*	*r*
Clinical Stage	<0.001	0.524	<0.001	0.690
Myeloma Bone Disease	<0.001	0.524	<0.001	0.690
Bone marrow plasmocytosis	0.004	0.346	0.011	0.39
β2-microglobulin*	<0.001	0.577	<0.001	0.543
LDH	0.001	0.397	0.015	0.299
CRP	NS	-	0.024	0.277

**Patients without RF.*

RANKL/OPG ratio was also significantly higher in MM compared to controls: 0,125± 0,019 vs 0,053±0,019; (p<0,001). The highest levels of RANKL and RANKL /OPG ratio were found in patients with MBD grade 2+3: 0,589±0,076 and 0,185±0,03 respectively. RANKL and RANKL /OPG ratio correlated strongly with clinical stage, MBD, bone marrow plasmocytosis, LDH and β2-microglobulin in the group without RF (Table). No correlation was found between RANKL, RANKL/OPG and the immunological variant, serum levels of Ca, creatinin, haemoglobin and albumin. *Conclusios*. Our data show that OPG, RANKL and RANKL/OPG ratio are important clinical markers of MBD and a future target for therapeutic intervention. Increased OPG levels in patients with RF are most probably a result of compensatory reaction to the increased bone resorbtion, rather than to the reduced glomerular filtration.

0255

SAFETY AND EFFICACY OF BORTEZOMIB FOR RELAPSED/REFRACTORY MULTIPLE MYELOMA IN DAILY ONCOLOGY PRACTICE

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Background. Bortezomib is a novel first-in-class anti-cancer agent, a proteasome inhibitor. Several publications reported on the safety and efficacy of bortezomib in the treatment of relapsed/refractory multiple myeloma in clinical trials. Complementary data on the experience with bortezomib in daily oncology practice are needed. *Aim.*Evaluate the safety and efficacy of bortezomib in the treatment of relapsed/refractory multiple myeloma in routine clinical practice. *Methods.* Patients having undergone >2 prior lines of therapy were treated within a Compassionate Use Programme proposing 6 cycles of bortezomib. While treatment modalities were at physician's discretion, the Programme recommended 1.3 mg/m^2 i.v. bortezomib on days 1, 4, 8 and 11 of each 21-day cycle. Addition of oral dexamethasone (20 mg the day of and the day after bortezomib administration) was recommended after 2 or 4 cycles in case of PD or SD, respectively. Post-hoc safety and efficacy analysis of patient records was performed using predefined criteria. Best response achieved was evaluated by the attending physician or by marker (M-protein or light chain) nadir level. Responders were patients with at least MR (a decrease ≥25% in marker level vs. baseline). CR/nCR was defined by physician's criteria or by a decrease >95% in marker level vs. baseline. Patients that received <2 cycles and irregular injections were excluded from the main analysis. However, a response analysis on an intention-to-treat (ITT) basis also included these patients. *Results.* Eighty-eight patients entered the Programme involving 62 oncologists/haematologists. Data from 5 patients were unavailable. Main analysis focused on 69 patients and ITT response analysis included 83 patients. Median patient age was 66 years [44-86], median time since diagnosis was 4 years [0.5-14] and median number of previous treatments was 3 [2-6]. Median number of bortezomib cycles at data collection was 4 [2-19] and 37.9% of patients were co-treated with corticoids during the Programme. In 73.9% of patients (51/69) at least an MR was observed (61.4% in the ITT analysis). In 37.7% of cases, this response occurred within the first cycle and in 95.5% within 3 cycles. Best response was achieved within the first cycle in 11.1% of patients and within 3 cycles in 68.9%. In the other responders, continuation of treatment improved the quality of response. Eighteen weeks after treatment initiation, corresponding to the anticipated duration of the Programme, 76.2% of the responders were still responding (≥MR). At the time of data collection, median 4.75 months [0.5-13] after last bortezomib injection, 44.9% of patients (31/69) were still responding (37.3% of ITT patients), according to the physician's evaluation. The most frequently reported adverse events were peripheral neuropathy (34.3%), thrombocytopenia (29.9%), diarrhoea (23.9%) and fatigue (22.4%). Among the cases of peripheral neuropathy, 65.2% were due to aggravation of a pre-existing condition. No cases of bortezomib-related haemorrhage were reported. *Conclusions.* The use of bortezomib in daily clinical practice resulted in encouraging high response rates with a predictable adverse event profile in patients with relapsed/refractory multiple myeloma. Efficacy and safety data were similar to those reported in clinical trials.

0256

MULTIPLE MYELOMA (MM) IN BRAZIL: CLINICAL AND DEMOGRAPHIC FEATURES AND THE UTILITY OF ISS IN 1,017 PATIENTS, MOSTLY WITH ADVANCED DISEASE

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Purpose. No large studies have described the features of MM in Brazil. Our aim was to characterize the demographic and clinical features of patients with MM treated at tertiary care centers, and to assess the predictive accuracy of the International Staging System (ISS) in such patients. *Methods.* 15 hematology centers provided information on patients diagnosed between 1998 and 2004, whose data were obtained from institutional charts and entered on a web-based system designed for the study. Chi-square tests were used for proportions, and survival was analyzed by the Kaplan-Meier method and log rank tests. *Results.* 1,017 patients (49.4% female), with ages ranging from 28 to 94 (median, 60.8) years, were evaluated. Race was white/mixed in 82.5%, black in 16.6%, and Asian in 0.9%. In 857 (84.2%) cases with immunoglobulin (Ig) isotype, it was IgG in 52%, IgA in 18.6%, light chain in 9.9%, non-secretory in 3.4%, and IgM in 0.3%. Bone lesions were present in 84.7% of patients, and Durie-Salmon staging (DSS) was I in 6.5%, II in 16.4%, and III in 77.1% of cases. At the end of February, 361 patients (35.5%) had died. Median follow up for the entire sample was 20.2 months, and 26.8 months for surviving patients. There was a significant difference between the overall survival (OS) of patients with DSS I, II and III (p<0.001), but there was considerable overlap between the curves for stages I and II. Median OS for DSS I, II and III was not reached, 65.7, and 49.3, respectively. Among 934 patients with complete data, ISS category was I in 134 (14.3%), II in 583 (62.5%), and III in 217 (23.2%). The median OS was not reached, 57.5 and 24.6 months for these three groups, respectively (p<0.001). After 5 years, the estimated OS for patients with ISS I was 68%. 237 (23.3%) patients underwent high-dose chemotherapy (HCT), and had a median OS of 77.2 months, compared with 39.4 months for those not receiving HCT (p<0.001). Other significant factors for OS in univariate analyses were hypercalcemia (p<0.001) and elevated LDH (p=0.028). Hypercalcemia was found in 10.7% of patients with ISS I, 23.3% in ISS II, and 34.0% in ISS III (p<0.001). Inspection of the OS curves shows ISS to be more useful than DSS in this sample with mostly advanced disease, with better segregation of the groups and nominally smaller P values than seen with DSS. *Conclusion.* This retrospective study confirms the prognostic utility of ISS, and suggests that ISS is more useful as a prognostic indicator in a sample of patients diagnosed in late stages of the disease, as determined by the DSS.

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0257

EVALUATION OF 4 STAGING SYSTEMS IN 470 MYELOMA PATIENTS: VALIDATION OF THE SUPERIOR PROGNOSTIC SIGNIFICANCE OF THE INTERNATIONAL STAGING SYSTEM

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Background. The wide range in survival rates in multiple myeloma (MM) establishes the necessity for a staging system with prognostic reliability. For more than 30 years, the Durie-Salmon Staging System (DSSS) remained the system of reference despite its drawbacks. In the meantime, the prognostic significance of the combination of β2 microglobulin (β2m) and albumin (alb) has been recognized and subsequently employed by Bataille *et al.*, the South West Oncology Group (SWOG) and most recently the International Myeloma Working Group (IMWG), in attempts to develop simpler staging systems with a stronger prognostic impact. *Aims.* To evaluate and compare the prognostic significance of these 4 staging systems in a large number of previously untreated MM patients. *Methods.* Between January 1989 and January 2006, 470 consecutive patients were diagnosed with MM in our department. Ninety-two (19.6%) patients received high dose therapy followed by autologous stem cell transplantation and the rest 378 (80.4%) were treated with conventional chemotherapy. All patients were classified according to the following staging systems: 1) DSSS. 2) Staging System of Bataille *et al.* (BSS). Stage I: β2m <6 mg/L and alb >3 g/dL. Stage II: β2m ≥6 mg/L and alb >3g/dL. Stage III: alb ≤3g/dL. 3) Staging System of the SWOG (SWSS). Stage I: β2m <2.5mg/L. Stage II: 2.5 mg/L ≤β2m <5.5 mg/L. Stage III: β2m ≥5.5mg/L and alb ≥3g/dL. Stage IV: β2m ≥5.5mg/L and alb <3g/dL. 4) International Staging System (ISS) of the IMWG. Stage I: β2m <3.5mg/L and alb ≥3.5g/dL. Stage II: neither stage I nor III. Stage III: β2m ≥5.5 mg/L. Overall survival (OS) was estimated according to Kaplan-Meier method. Differences in survival were assessed using the log-rank test. *Results.* The distribution and median OS of the patients according to each staging system are displayed in Table 1. Classification according to DSSS, BSS and SWSS yielded a significantly heterogeneous distribution of our patients, with the majority being classified in stage III, I and II respectively. ISS achieved the most homogeneous patient distribution. There was no statistically significant difference (p<0.05) in survival between stages II and IIIA of DSSS, II and III of BSS, as well as between stages III and IV of SWSS. ISS alone yielded significant difference (p< 0.0001) in survival between all three stages. *Conclusion.* Our study confirms the superiority of ISS over DSSS and previous prognostic classifications based on the combination of β2m and albumin. ISS proved to be a simple, reproducible alternative with high prognostic power, definitely able to gain wide clinical applicability.

Table 1.

	DSSS		BSS		SWSS		ISS	
Stage	N (%)	OS months (95% CI)	N (%)	OS months (95% CI)	N (%)	OS months (95% CI)	N (%)	OS months (95% CI)
I	38 (8.1)	75 (62-78)	270 (57.4)	53 (44-62)	73 (15.5)	76 (67-85)	135 (28.7)	76 (66-86)
II	131 (27.9)	47 (37-57)	79 (16.8)	25 (21-31)	229 (48.7)	45 (41-49)	168 (35.7)	40 (35-45)
III	301 (64)	36 (32-40)	121 (25.7)	19 (13-23)	98 (20.8)	23 (18-28)	167 (35.5)	23 (19-27)
IIIA	231 (49)	38 (35-41)						
IIIB	70 (15)	24 (16-32)						
IV					70 (14.8)	21 (15-27)		

0258

OSTEONECROSIS OF THE JAW IN PATIENTS WITH MULTIPLE MYELOMA TREATED WITH BISPHOSPHONATES: A SINGLE CENTER EXPERIENCE

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Background. Bisphosphonate (BP)- associated osteonecrosis of the jaw (ONJ) is a new and distinct clinical entity. Cases of ONJ associated with the administration of BPs, that is characterized by dehiscence of the oral mucous membranes and exposure of necrotic underlying mandible or maxilla, were first reported in 2003. *Aim.* To study retrospectively a large number of multiple myeloma (MM) patients treated with BPs, in order to estimate the incidence and identify possible risk factors for the development of ONJ. *Methods.* Administration of BPs was initiated in our department in 1991. A review of the medical records of all patients diagnosed with MM since 1991 was performed. We evaluated the type of BP administered, the time of exposure to BP and the cases of ONJ. The diagnosis of ONJ was based on the presence of symptoms and signs of intraoral bone necrosis, the findings of panoramic x-rays and the results of bone biopsies. Patients were divided into 3 groups according to the type of BP administered. Group A received pamidronate, Group B zoledronate and Group C pamidronate and zoledronate sequentially. The $\chi 2$ test was used for comparisons of proportions across levels of categoric variables. Mann Whitney U test and One Way ANOVA test were used to compare the median and mean time of exposure to BPs among groups respectively. Kaplan Meier method was used to estimate the actuarial risk of ONJ in each group. Differences were assessed using the log-rank test. Throughout the analysis a level of 5% was used to denote statistical significance. *Results.* Between 1991 and 2005, 303 patients with MM were diagnosed in our department. Bisphosphonates were administered to 254 (83.8%) patients with median time of exposure 15 months (4-77). Group A included 78 patients (30,7%), Group B, 91 (35,8%) and Group C, 85 (33,5%) with median time of exposure 10 (4-38), 12 (4-52) and 36 (6-77) months respectively.(pA,C <0.000, pB,C<0.000). Forty- nine (16,2%) patients did not receive BPs. Twenty eight cases (11,02%) of ONJ were observed among patients treated with BPs. None of the patients without exposure to BPs developed ONJ. The median time of exposure to BPs in patients who developed ONJ was 35 months (12-68), whereas the respective time for patients who did not, was 14 months (4- 77) (p<.001). One case of ONJ was observed in group A (1,28%), 6 cases in group B (6,5%) and 21 in group C (24,7%). (pA,B =0.084). All ONJ cases in Group C occurred during treatment with zoledronate. The actuarial risk of ONJ after 18 months of administration was 3,7% for group A and 7,8% for group B (p=0.25). At 36 months the actual risk of ONJ was 37,2% and 12,4% for group B and C respectively (p=0.036). *Conclusions.* The incidence of ONJ in patients with MM treated with BPs is high. Time of exposure and probably the type of BP seems to contribute to the occurrence of ONJ.

Chronic lymphocytic leukemia and related disorders – Clinical/Experimental I

0259

DETECTION OF RISK-IDENTIFYING MARKERS AND ADDITIONAL ABERRATIONS BY MULTIPLEX LIGATION-DEPENDENT PROBE AMPLIFICATION (MLPA) IN CHRONIC LYMPHATIC LEUKEMIA

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B cell chronic lymphatic leukemia (B-CLL) is the most common form of leukemia in adults. Recently, deletions of ATM, TP53 and trisomy of chromosome 12 have been identified as unfavorable markers using interphase FISH. FISH analysis is labor-intensive, expensive and limited to the number of probes analyzed. We developed a robust method based on multiplex ligation-dependent probe amplification (MLPA) of target sequences for 40 different tumor-associated genes, including unfavorable risk-identifying deletions of ATM and TP53 and trisomy of chromosome 12. MLPA data of 53 cases with CLL and one case with follicular lymphoma (FL) were validated using conventional karyotyping and interphase FISH analysis, revealing high sensitivity and specificity of the assay for these risk-identifying mutations. DNA profiling using MLPA identified recurrent gain of PMAIP1 and BCL2 (18q21.3), known targets in B-cell non-Hodgkin lymphoma (NHL). A recurrent deletion of CDNK2A/B locus (9p21) was found, that was associated with aggressive disease progression. A trisomy chromosome 19 was identified that went undetected by cytogenetics. MLPA confirmed trisomies of chromosomes 7 and 15. Here we demonstrate that MLPA can be used for rapid analysis of known risk-identifying markers and detection of additional numerical cytogenetic unbalances in B-CLL.

0260

QUANTITATIVE GENE EXPRESSION ANALYSIS OF SURROGATE MARKERS FOR GENETIC RISK GROUPS AND SURVIVAL IN CLL

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Background. The genetic factors VH mutation status, V3-21 gene usage, and genomic deletions at 11q22-q23 and 17p13 have been shown to be important prognostic markers in CLL. Given the high complexity of these analyses in the recent years several molecular surrogate markers were developed aiming at the facilitation of routine prognosis assessment. *Aims.* To assess the value of potential surrogate markers for the prediction of genetic risk groups and survival. *Methods.* Real-time RT-PCR (RQ-PCR) of candidate genes was performed in a CD19-purified and a non-purified CLL cohort each comprising the relevant genetic subgroups (VH mutated, VH unmutated, V3-21 usage, 11q-, 17p-). 17 markers (ADAM29, ATM, CLLU1, DMD, GLO1, HS1, KIAA0977, LPL, MGC9913, PCDH9, PEG10, SEPT10, TCF7, TP53, Vimentin, ZAP-70, ZNF2) identified in previous studies were investigated in the non-purified cohort of 102 CLL patients. Of these, 10 markers, either with an overexpression in non-CLL cells or an impact on survival or risk group prediction, were analyzed in the purified cohort of 112 cases. VH sequencing and FISH screening for genomic aberrations were carried out for all cases. Survival information was available for 80 (purified) and 88 cases (non-purified). Logistic regression was performed to test the predictive value of gene expression for genetic risk groups, Cox proportional hazards statistics for survival analysis. *Results.* The genetic risk groups in both cohorts showed the expected correlation with survival with significantly shorter survival of VH unmutated, 17p-, and 11q- cases indicating a representative composition of the cohorts under study. In non-purified cases, the best predictive marker for VH status was LPL (p=0.001). While no reliable predictive markers were identified for V3-21 usage or 17p-, lower ATM expression was predictive for 11q-. In survival analysis including all candidate genes, TCF7 (p=0.001) and KIA0977 (p=0.016) were of prognostic value. In multivariate survival analysis including candidate gene expression and the genetic risk factors as variables, only 17p- remained as a significant parameter. In the purified cohort, significant markers (p<0.05) for genetic risk groups were: ZAP70, LPL, and TCF7 for VH mutation status (TCF7 expression associated with mutated VH); SEPT10, ZAP70, and ADAM29 for V3-21 usage; ATM and TCF7 for 11q- (both with a negative association); ZNF2 for 17p- (negative association). In survival analysis including the expression of all candidate genes, only TCF7 was identified as a significant factor. In contrast to ZAP70, which was of borderline significance (p=0.061), TCF7 expression was positively correlated with survival times. In multivariate analysis, the parameters 17p-, 11q-, V3-21 usage, TCF7 and ZAP70 expression were identified as independent prognostic factors. *Summary/Conclusions.* Several results obtained in CD19-purified cases could not be reproduced in unpurified cases strongly arguing for a tumor cell selection prior to expression analysis. In purified cases, ZAP70, LPL, and TCF7 were the best predictors for VH mutation status. Additional markers such as ATM and ZNF2 may help to identify genomic risk groups such as 11q- and 17p-. Multivariate survival analysis suggests TCF7 as a strong survival predictor and points to a pathogenic role for this gene in CLL.

0261

DISTINCT EXPRESSION LEVELS OF NOXA IN PERIPHERAL VERSUS LYMPH NODE CHRONIC LYMPHOCYTIC LEUKEMIA CELLS ARE LINKED WITH SURVIVAL CAPACITY

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Background. The relentless accumulation of chronic lymphocytic leukemia cells is presumed to derive from proliferation centers in lymph node and bone marrow. To what extent the properties of these leukemic cells are linked with the often stated characteristic anti-apoptotic phenotype of CLL is unknown. Recently, we have described that in peripheral blood CLL samples, aberrant apoptosis gene expression was not limited to protective changes but also included increased levels of pro-apoptotic Noxa and Bmf. The functional consequence of this finding is not known, nor whether this aberrant apoptosis gene profile is also present in CLL proliferation centers. *Aim.* To perform a functional comparison of apoptosis gene profiles from peripheral blood CLL versus lymph node proliferation centers. *Methods*. Immunofluorescence microscopy, RT-Multiplex-Ligation-dependent Probe Amplification (RT-MLPA), Western blot, Transfection, RNA interference. *Results.* Lymph node material from 9 B-CLL patients and peripheral blood samples from 16 B-CLL patients were included. All B-CLL expressed CD5, CD23 and CD19 or CD20. Over 90% of the lymph nodes consisted of lymphocytes. Ki67+ cells were either scattered throughout the lymph nodes or in follicle-like structures. RNA samples were subjected to the RT-MLPA procedure which monitors expression of 34 apoptosis genes. Apart from expected differences in survivin and Bcl-Xl, the most prominent distinction with peripheral CLL cells was the generally low levels of Noxa in lymph node samples. A reduction in Noxa RNA and protein levels could also be obtained by *in vitro* stimulation of peripheral blood CLL with CD40. Direct manipulation of Noxa protein levels was achieved by proteasome inhibition in CLL and via RNAi in model cell lines. In all these instances, the viability of the cells was directly linked with Noxa levels. *Conclusions*. These data indicate that *spontaneous* apoptosis of peripheral CLL cells *in vitro* is linked with high Noxa levels. We propose that suppression of Noxa in the lymph node contributes to the persistence of CLL, and that therapeutic targeting of Noxa might be beneficial.

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0262

SIGNIFICANT CORRELATION BETWEEN OBJECTIVE RESPONSES AND EXPOSURE TO HUMAX-CD20 IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. The fully human monoclonal IgG1 antibody HuMax-CD20 targets a novel epitope of the CD20 molecule on B-cells. HuMax-CD20 stops growth of engrafted B-cell tumors in SCID mice more effi-

ciently than Rituximab and i.v. infusion of HuMax-CD20 in cynomolgus monkeys leads to profound, long lasting, dose-dependent B-cell depletion. *Aim.* The objective of the present trial was to establish the safety, efficacy and the pharmacokinetics of HuMax-CD20 in patients with chronic lymphocytic leukemia. *Methods.* Data are presented from an open label, dose-escalation, multicenter phase I/II clinical trial. 3 cohorts of 3 (A), 3 (B) and an extended cohort of 27 (C) patients with relapsed or refractory chronic lymphocytic leukemia (B-CLL) received 4 weekly i.v. infusions of HuMax-CD20 and will be followed for 12 months. The first infusion was 100 mg, 300 mg and 500 mg in cohort A, B and C and the following 3 infusions were of 500, 1000 and 2000 mg, respectively. Patients were premedicated with oral acetaminophen and i.v antihistamine and received i.v. glucocorticoids before first and second infusions. The endpoints were B-cell depletion, adverse events, objective response according to the NCI working group guidelines for CLL, time to progression, duration of response, time to next anti-CLL treatment, and pharmacokinetics. *Results.* Median age was 61 years; median time since diagnosis was 6.3 years. Maximum tolerated dose was not reached. All patients in the highest dose group had pronounced reduction of the leukemic CD19+CD5+ cell counts. Objective response rate was 46% (12 of 26 evaluable patients in cohort C) with 2 nPR and 10 PR. By analyzing pharmacokinetic parameters, a statistically significant increased AUC were demonstrated in responders (median: 1356 µg/mL*h, range: 965-1830) compared to non-responders (median: 940 µg/mL*h, range: 540-1260), p=0.011. Similar significant differences were found for Cmax and Cmin. *Conclusion.* This preliminary analysis of data from the first 33 CLL patients treated with HuMax-CD20 demonstrated significant depletion of CD19+CD5+ cells and provided an indication of clinical efficacy that correlates with the exposure to HuMax-CD20. These data encourage further development of HuMax-CD20 in CLL.

0263

CD40 LIGATION SENSITIZES P53 DYSFUNCTIONAL CLL CELLS TO CHEMOTHERAPY INDUCED APOPTOSIS VIA THE P73 PATHWAY

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CD40 activation of chronic lymphocytic leukemia (CLL) cells enhances their capacity to induce an immune response. Although CD40-activation has been shown to enhance sensitivity to both cytotoxic T cell mediated killing and death receptor mediated apoptosis, its effect on chemotherapy is much less clear. We showed recently that CD40 activation of CLL cells resulted in induced expression of pro-apoptotic factors like death receptors, p21 and the BH3-interacting-domain death agonist (Bid), even in CLL cells with dysfunctional p53. Since the effect of fludarabine is highly mediated by p53 dependent response genes we hypothesized that CD40 activation could sensitize p53 dysfunctional CLL cells to fludarabine mediated apoptosis. In *ex vivo* studies, we show that stimulation of CLL cells by co-culture with CD154-expressing cells induced leukemia-cell expression of p73, a p53-related transcription factor that is regulated by the c-Abl tyrosine kinase in both p53 functional and dysfunctional cases. Transduction of CLL cells with an adenovirus encoding p73 also induced Bid. Next we showed that p53-dysfunctional CLL cells resistant to fludarabine treatment could be sensitized to fludarabine upon CD40 ligation or p73 transduction. This phenomenon could be suppressed by specific c-abl inhibition through imatinib treatment. These results demonstrate that CD40 ligation may sensitize leukemia cells not only to extrinsic but also intrinsic apoptotic stimuli via a c-Abl-dependent pathway and that CD40-based therapy may be helpful in overcoming the resistance of p53-dysfunctional CLL to anti-cancer therapy.

0264

RHAMM/CD168 IS A NOVEL LEUKEMIA ASSOCIATED ANTIGEN WITH PROGNOSTIC VALUE FOR PATIENTS WITH B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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Background and Aims. Differential expression of molecules in patients with B-cell chronic lymphocytic leukemia (B-CLL) might define suitable targets for T cell based vaccines and/or antibody approaches. *Methods.* We assessed the mRNA expression of the tumor associated antigen (TAA) RHAMM/CD168 defined earlier by serological analysis of cDNA expression libraries (SEREX) from leukemic cells. *Results.* Peripheral blood mononuclear cells from 40 B-CLL patients and 20 healthy volunteers (HVs) were examined by quantitative RT-PCR. A leukemia-restricted expression of the antigen RHAMM/CD168 was observed in 39/40 B-CLL patients in contrast to the absence of its expression in HVs. To evaluate the immunogenicity of this novel LAA, mixed lymphocyte peptide cultures (MLPCs), followed by enzyme-linked immunosorbent spot (ELISPOT) and flow cytometry assays were performed to detect antigen-specific CD8+ T cells. RHAMM/CD168 specific responses by CD8+ HLA-A2/R3tetramer+CCR7-CD45RAhigh effector T cells were detected. As these CD8+ T cells contribute to the elimination of RHAMM+ CLL cells, we questioned whether expression of the antigen would be associated with a better survival. RHAMM/CD168 expression revealed to be higher in patients with unmutated IgVH status. RHAMM normalized against the housekeeping gene TATA binding protein (TBP), i.e. the RHAMM/TBP ratio was defined as a prognostic surrogate marker for B-CLL. B-CLL patients with a RHAMM/TBP ratio > 1.38 showed a significantly shorter treatment free survival (TFS). A tendency towards higher RHAMM/TBP expression ratios was observed in B-CLL cases with del11q. *Conclusion.* RHAMM/CD168 is a novel LAA in B-CLL patients, an antigen correlating with the clinical course of the disease. Therefore, we consider RHAMM/CD168 an interesting target for immunotherapy in early stage B-CLL patients, especially with worse prognosis (IgVH unmutated).

0265

PARTHENOLIDE INDUCES REACTIVE OXYGEN SPECIES AND SELECTIVE APOPTOSIS OF B-CHRONIC LYMPHOCYTIC LEUKAEMIA CELLS VIA A P53 INDEPENDENT PATHWAY

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Background. CLL is incurable using conventional therapy. While the disease is controlled by treatment with chlorambucil (CHL) or fludarabine (FLU), extended exposure results in resistance, which may result from impaired operation of the p53-mediated apoptotic pathway. It is therefore important to identify novel agents which rapidly and selectively induce p53-independent apoptosis of cells isolated from patients resistant to conventional drugs. Parthenolide (PTL) is the active component of feverfew which has been extensively used for the treatment of migraines. PTL induces apoptosis via reactive oxygen species (ROS) generation and blockade of components of the potent anti-apoptotic NF_B pathway, including the upstream regulator I_B kinase. *Aims.* To determine (a) whether PTL is selectively toxic to CLL cells, (b) whether PTL is toxic to CLL isolates resistant to conventional drugs and (c) whether its cytotoxic mechanism involves ROS generation and NFκB inhibition. *Methods.* Western blotting, MTT dye reduction assays and morphological analysis of cytospin preparations were employed to quantify apoptosis and related cell death. ROS production was measured using FACScan analysis of cells treated with CM-H2DCFDA, which reacts with ROS to produce the fluorescent compound deacetylated H2DCF. Induction of a pro-apoptotic conformation change of the Bax protein was quantified by immunoprecipitation using a conformation-specific antibody (Pharmingen). *Results.* We investigated the sensitivity of isolates from 78 CLL patients, to PTL and CHL. The median LD50 for PTL was 6.2µM. Fifteen isolates which were resistant to CHL (LD50 >300µM) were readily killed by PTL concentrations <15 µM. Fifteen of these isolates were relatively resistant to the conventional agent CHL but retained sensitivity to PTL. Brief exposures to PTL (1-3h) were sufficient to induce caspase activation and cell death. In contrast, cell killing was not detected until >12h exposure to 75 µM CHL. CLL cells were strikingly more sensitive to apoptosis induction by 2-7.5 µM PTL than were normal T or B lymphocytes or CD34+ bone marrow progenitor cells. In contrast to CHL, PTL failed to elevate p53-levels, suggesting a p53-independent mode of action. This conclusion was confirmed by the observation that pifithrin α, an inhibitor of p53-mediated transcription, failed to abrogate cell killing by PTL. Mechanistic studies showed that killing of B-CLL cells by PTL was via PTL-induced generation of ROS, since treatment with N-acetylcysteine (NAC) abrogated the PTL induced apoptosis. ROS generation resulted in turn in a pro-apoptotic Bax conformational change, release of mitochondrial cytochrome c and caspase activation. PTL also decreased nuclear levels of the anti-apoptotic transcription factor NF_B and diminished phosphorylation of its negative regulator I_B. This is the first report showing the relative selectivity of PTL towards CLL cells and its p53-independent mechanism of apoptosis induction. *Conclusion.*

In conclusion, the rapid, selective, p53-independent cytotoxic action of PTL and its ability to kill CLL isolates refractory to cytotoxic drugs suggest that this agent may be of value in the treatment of CLL patients resistant to conventional regimes. These data also suggest that further investigation of natural products that induce ROS may be of potential therapeutic use for the treatment of B-CLL.

0266

SMALL MOLECULE PAN-BCL2 FAMILY INHIBITOR OBATOCLAX (GX15-070): FINAL RESULTS OF A SINGLE AGENT PHASE I TRIAL IN PATIENTS WITH REFRACTORY CHRONIC LYMPHOCYTIC LEUKEMIA (CLL)

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Background. Obatoclax is a synthetic small molecule which inhibits the binding of the antiapoptotic proteins bcl-2, bcl-xl, bcl-w and mcl-1 to the proapoptotic proteins bax and bak. It shows broad single agent cytotoxicity in cancer cell lines *in vitro* and *in vivo* and in human B-CLL cells *ex vivo*. Bcl-2 family proteins are universally overexpressed in CLL in which accumulation of malignant cells is thought to be the direct result of the consequent inability to undergo apoptosis. *Aims.* determine safety profile, recommended phase II dose, pharmacokinetic and pharmacodynamic profile of obatoclax in refractory CLL. *Methods.* accelerated dose escalation of 1-3 h infusions every 3 weeks with single intra-patient dose escalation allowed. To assess obatoclax's direct effect on the release of proapoptotic proteins, the relative levels of activated bak/bax hetero oligomers were monitored serially in PBMNCs following dosing. Induction of apoptosis was monitored quantitatively with serial determinations of plasma concentration of histone-oligonucleosomal DNA (ODNA) complexes. *Results.* 12 patients enrolled at doses ranging from 3.5-14 mg/m^2 administered using a 1 hr infusion and 13 patients at 20-40 mg/m^2 using a 3 hr infusion. Median age was 61 (range 46-76), Rai stage was III-IV in 19 and median number of prior therapies was 4 (range 2-10). The most frequent adverse events (AE) have been somnolence grade 1 (40%) or 2 (19%) and euphoria grade 1 (47%) or 2 (9%) occurring during or shortly following the infusion. Other AE's reported in ≥25% of patients were transient O2 desaturation (25%), AST increase (34%) and fatigue (34%). Dose-limiting toxicities have been Grade 3 infusional neurological events such as somnolence, ataxia and dysphoria. Doses ≥10 mg/m^2 were associated with a significant increase in activated bak/bax hetero oligomers sustained for up to 8 hrs at the higher doses evaluated. An early release of ODNA occurred 1-6 hrs after the start of the infusion. A secondary increase occurred with a noticeable lag time from the peak plasma GX15-070 concentration (24 to 168 hrs after the start of the infusion). There was a correlation between peak plasma ODNA concentration (median = 400 range 0-4358 AU/mL) and dose (threshold effect at 14 mg/m^2) as well as AUC (max ODNA 2x baseline if AUC<180 ng.hr/mL vs. 15 x baseline when AUC≥180 ng.hr/mL; p<0.015). 18/25 patients showed reduction of peripheral lymphocyte counts (mean of 29%). Best clinical responses assessed by CLL Workshop Criteria so far are : unconfirmed PR in 1. In addition, 4/14 patients with baseline platelet count <100,00/mm^3 showed sustained elevations of platelet counts by ≥50% including two patients improving from 70,000 to 144, 000/mm^3 and 47,000 to 105,000/mm^3; 3/11 patients who were anemic at baseline showed sustained elevations of Hb from 8.7 to 10.6 g/dL, 7.9 to 13.9 g/dL and 8.7 to 9.8 g/dL, the latter two achieving transfusion independence. *Conclusions.* single agent obatoclax exhibits dose dependent biological activity in refractory CLL with documented induction of apoptosis and improvement in hematological parameters at well tolerated doses. The recommended phase II dose is 28 mg/m^2 using a 3 h infusion.

0267

COMPARISON OF *EX VIVO* DRUG SENSITIVITY BY TRAC ASSAY AND PATIENT RESPONSE IN THE UK LRF CLL4 TRIAL

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Background. Previous results have suggested that *ex vivo* drug sensitivity test results are independent prognostic or predictive factors for subsequent patient response in chronic lymphocytic leukaemia. *Aims.* In order to determine its accuracy at predicting response and survival, drug sensitivity is being tested both at initial entry (closed October 2004) and at second randomisation (still open) in the UK Leukaemia Research Fund (LRF) CLL4 trial. *Methods.* At first randomisation, blood specimens were sent to Bath for drug sensitivity testing: initially by DiSC (Differential Staining Cytotoxicity) assay; subsequently by its development, the TRAC (Tumour Response to Anti-neoplastic Compounds) assay. Ten drugs were tested including chlorambucil, fludarabine and mafosfamide (used *in vitro* in place of cyclophosphamide). LC90s were calculated. Patients were randomised into Trial arms to receive chlorambucil (Chl), fludarabine (Flu) or fludarabine+cyclophosphamide (FluCy) in the ratios 2:1:1. Numbers of patients with any versus no response were compared. 2P is by Fisher's exact test. *Results.* From 777 randomised, LC90 results from 442 patients could be compared with subsequent patient response. Definitions of test-sensitive were LC90s of ≤6.3 ug/mL for chlorambucil, and ≤10.0 ug/mL for both fludarabine and mafosfamide. No difference in average drug sensitivity was found between Trial arms. Results are presented in the Table. All differences between response rates in the test sensitive and resistant groups were highly statistically significant. For instance, for those treated with Flu or FluCy, 90.7% (95% confidence interval (CI) = 86.8-94.6) of test-sensitive patients responded compared with 22.2% (3.0-41.4) test-resistant patients. *Conclusions.* At diagnosis of CLL, even within a group of patients with a high clinical response rate, *ex vivo* drug sensitivity can be used to identify a proportion of patients with a significantly poorer probability of clinical response. TRAC results predict better for patient response to fludarabine (± cyclophosphamide) than for response to chlorambucil.

Table 1. Comparison of ex vivo drug sensitivity with subsequent patient response (numbers of patients).

	Test sensitive		*Test resistant*			
Trial arm	*No. Response*	*No Response*	*Response*	*No Response*	*Odds ratio (95% CI)*	*2P*
Chl	*210 141*	*45*	*9*	*15*	*5.2 (2.1-13)*	*0.0004*
Flu+FluCy	*232 194*	*20*	*4*	*14*	*34.0 (10-1113)*	*<0.00001*
Total	*442 335*	*65*	*13*	*29*	*11.5 (5.7-23)*	*<0.00001*

0268

INTRACELLULAR CYTOKINE EXPRESSION BY B AND T CELLS DIFFERS IN ZAP-70 POSITIVE AND ZAP-70 NEGATIVE B-CLL PATIENTS

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Background. Changes in cytokine network between malignant cells and residual T lymphocytes may be responsible for accumulation of malignant cell clone and for immune abnormalities in B-cell chronic lymphocytic leukemia (B-CLL). B-CLL is the most frequent type of adult leukemia in Western countries and it seems to be a heterogeneous disease with a highly variable clinical course and prognosis. Recently the role of ZAP-70 (zeta associated protein, a member of the Syk-ZAP-70-protein tyrosine kinase family) as a surrogate marker for IgVH mutation identifying patients with a more aggressive clinical course was reported and it seems to be the most important prognostic factor in B-CLL. *Aims.* In this study we tried to determine whether cytokine production changed according to ZAP-70 expression. We assessed the expression of IL-2, IL-4, IFN-γ and TNF-α by CD19 and CD3 cell subsets in group of ZAP-70 positive and ZAP-70 negative B-CLL patients. *Methods.* We analyzed the blood samples obtained from sixty newly diagnosed, untreated B-CLL patients. Peripheral blood lymphocytes were isolated and stimulated by PMA and ionomycin in the presence of Brefeldin A to assesses intracellular cytokine expression. Then combined membrane and intracytoplasmic staining procedures were performed using fluorochrome-conjugated monoclonal antibodies. All samples were also stained for ZAP-70 protein expression by CD19+/CD5+ cells. The multi-color flow cytometry technique was used to analyze labeled cells. The data were shown as percentage of analyzed cell subset and mean fluorescence intensity (MFI) indicating the level of cytokine expression by particular cell. *Results.* The mean percentage of CD3 cells expressing all analyzed cytokines was significantly higher in ZAP-70 positive in

comparison to ZAP-70 negative group. Such a difference was observed in MFI values, however it was not statisticlly significant. The mean percentage of CD19/TNF-α cells was significantly higher, while the percentage of CD19/IL-2, CD19/IL-4 and CD19/ IFN-γ was lower in ZAP-70 positive than ZAP-70 negative patients. There was no significant difference between ZAP-70 positive and ZAP-70 negative group as far as MFI was concerned. *Conclusions.* Our findings demonstrate an association between ZAP-70 and cytokine expression in B-CLL. The more aggressive course of disease is connected with higher capability of T cells in production of cytokines responsible for disease pathogenesis. Such a connection is also observed in production of TNF-α by malignant B lymphocytes. Our results may approve the role of ZAP-70 expression by malignant cells as a good prognostic marker for B-CLL.

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0269

ACCUMULATION OF T-CELL WITH EXTREMELY SHORT TELOMERES IN T-CELL PROLYMPHOCYTIC LEUKEMIA (T-PLL)

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Background. T-cell prolymphocytic leukemia (T-PLL) is a rare aggressive lymphoproliferative disease characterized by the expansion of a T-cell clone derived from immuno-competent post-thymic T-lymphocytes. Important mechanisms involved in expansion of human malignant cells are reactivation of telomerase, an enzyme complex, which is able to compensate the loss of telomere repeats by cell division, and maintenance or elongation of telomere length. *Aims.* Our aim was to investigate the role of telomeres in patients with T-PLL. *Methods.* We measured telomere length by automated multicolor flow-FISH and telomerase activity by telomeric repeat amplification protocol in subsets of peripheral blood leukocytes from 10 newly diagnosed or relapsed patients with sporadic T-PLL. *Results.* The average telomere length in the clonal T-cells of all samples analyzed was extremely short (mean±std : 1.6±0.6 kb) compared to the non-clonal T-cells (5.3±0.8 kb; p=0.012). The average telomere length for B-cells in these patients was 6.4±0.7 kb, n=5). Telomere length values of the clonal T-cells were all below the 1st percentile of telomere length values observed in T-cells from healthy aged-matched controls whereas non-clonal T-cells and B-cells fell between the 10th and 90th percentile of the normal distribution. In addition, we performed follow-up measurements of telomere length in one patient over a period of 18 months. Surprisingly, telomere length remained stably short at 1.0 kb±0.6 kb in the clonal T-cells without further telomere loss. No cell doublets indicative of fused or bridged chromosomes and telomere dysfunction were observed. Although telomerase is necessary to elongate or stabilize critical short telomeres levels of telomerase activity in the clonal T-cells were not higher than in controls. *Conclusions.* This is the first report of extremely short telomeres in clonal T-cells of patients with sporadic T-PLL. Our results are compatible with extensive proliferation of the clone. Most likely telomerase activity in T-PLL is sufficient to stably maintain extremely short telomeres and allow their clonal expansion. Current studies are aimed at exploring telomerase inhibitors to inhibit the proliferation of T-PLL cells and at the role of the very short telomeres in these cells regarding genomic instability and cytogenetic aberrations.

0270

INTRACLONAL DIVERSIFICATION OF IMMUNOGLOBULIN LIGHT CHAIN VARIABLE REGION GENES IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Analysis of somatic mutations in immunoglobulin heavy chain variable region (IGHV) genes in various types of B cell malignancies, including chronic lymphocytic leukemia (CLL), has demonstrated frequent intraclonal heterogeneity, indicating ongoing mutational activity. We recently showed that CLL light chain repertoire is skewed and characterized by CLL-biased features and also provided evidence for the complementary role of light chains in antigen recognition by CLL malignant cells. In the present study, we evaluated the intraclonal diversity status of IGKV/IGLV genes in 32 CLL cases; 25 cases expressed IgM/IgD, whereas 7 cases expressed IgG. IGKV-J and IGLV-J rearrangements were amplified by RT-PCR, purified, ligated into the pCRR 2.1 vector and transfected in *E. Coli*/TOP10F' cells. Sequence data were analyzed using the V-QUEST/IMGT and Clustalw/EMBL tools. Mutations observed in only one of the IGK/IGL molecular clones from the same sample were characterized as non-confirmed, whereas mutations observed more than once in the IGK/IGL molecular clones from the same sample were characterized as confirmed. The Taq DNA polymerase error rate in our laboratory is 0.052%, which may amount to 0.17 mutations/IGK or IGL clone. Overall, the cloning process was followed for 22 IGKV-J rearrangements (2/22 from lambda-expressing cases) and 10 IGLV-J rearrangements. Twelve out of 32 rearrangements (37.5%) carried IGKV/IGLV genes with greater than 98% homology to germline (*unmutated*); 5/12 *unmutated* IGKV/IGLV genes had 100% homology to germline. Information on the intraclonal variation was obtained by sequencing a minimum of 7 colonies per rearrangement. No differences were found between individual clones of 10/32 (31.2%) IGKV-J or IGLV-J rearrangements. The remaining rearrangements (22/32; 68.8%) exhibited intraclonal variation. The number of different subclones per cloning sample ranged from 3 to 5. Eight out of 32 rearrangements (25%) carried only non-confirmed mutations. Ten IGKV-J and four IGLV-J rearrangements (overall, 14/32; 43.8%) carried confirmed ongoing mutations. All nucleotide variations were single base substitutions, resulting in both silent (S) and replacement (R) mutations; nucleotide insertions or deletions were not observed. The number of nucleotide variations ranged from 1 to 7. Overall, 66 ongoing mutations (29 confirmed/ 37 non-confirmed) were observed: 24 S mutations and 42 R mutations. Twenty-nine out of 42 R mutations encoded for functionally similar amino acids. Most mutations were located in FR1/FR3/CDR1; occasional mutations were also detected in CDR2 and the IGK/L variable part of CDR3. Ongoing confirmed mutations were observed not only in *mutated* cases but also in 7/12 *unmutated* rearrangements, of which two had 100% homology. Mutations targeted A/G/C/T in a ratio of: 17/19/17/13. Transitions predominated over transversions (47 vs. 19); pyrimidines were targeted slightly more often than purines (36 vs. 30). These results indicate that IGK/IGL genes in CLL can undergo intraclonal diversification in a considerable percentage of cases and provide further support for the active contribution of light chains in antigen recognition. Mutations among subclones had specific molecular traits. Finally, intraclonal diversity did not correlate with the original mutational load, since it was observed both in CLL cases with little or no somatic mutations as in cases with considerable mutations.

0271

QUANTITATION OF MINIMAL RESIDUAL DISEASE IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA USING LNA-MODIFIED FLUORESCENTLY LABELED PROBES AND REAL-TIME PCR TECHNOLOGY

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Background. Patients with chronic lymphocytic leukemia (CLL) relapse even after aggressive therapies and stem cell transplantation. As the therapeutical goal today is to clear off the tumor cell burden as much as possible (by stem cell transplant or intensive chemoimmunotherapy), highly sensitive assays for minimal residual disease (MRD) evaluation and monitoring are needed. At present, many patients with not only germline IgVH sequences, but also with hypermutated IgVH genes are being treated, with the need for a sensitive and specific MRD monitoring. The original notion of MRD follow-up in CLL was based on the usage of JH-gene specific TaqMan hybridization probes. At present, due to the vast diversity of B-clonal rearrangements to be detected, the original idea has been challenged and the methodology should be modified. *Aims.* Since the hypermutation process does not restrict itself to the VH segments only and might afflict the JH segment as well, the molecular tools for the monitoring of B-CLL clonal rearrangements must be versatile enough to allow for the detection and quantitation of virtually any sequence possible. Moreover, the technique must meet the criteria for high sensitivity and specificity. We present here a novel methodology for MRD monitoring in CLL, based on LNA technology (Locked Nucleic Acids) and quantitative Real-Time PCR. *Methods.* Thirty-nine patients with the diag-

nosis of CLL were enrolled into our MRD monitoring study (16 females, 23 males, median age 59.6 yrs). 21 out of 39 individuals had unmutated IgVH genes (3 females, 18 males), 18 out of 39 patients had mutated IgVH genes (13 females, 5 males). For each patient, clone-specific primers were designed and their clonal IgVH sequences were molecularly cloned to construct the quantitation standards. In one patient, allelic inclusion has been identified (VH1-8 and VH3-30, both mutated), and for this individual, clone-specific primers and standards have been constructed for both rearrangements. To quantify the individual clonal IgVH transcripts, LNA-modified fluorescently labeled probes targeted against individual VH gene segments were employed. For any of 6 (7) IgVH families with unmutated IgVH genes, family-specific consensus LNA-modified probes were used. For those CLL cases with heavily hypermutated genes, ProbeLibraryTM was employed. For quantitation experiments, ABL was used as the control gene. *Results.* The LNA-modified probes are distinguished by a very high specificity and sensitivity (reaching to 10-8, in contrast to flow cytometry with its detection limit being 10-4). The LNA-based assays allow for precise monitoring of the residual tumor cell burden in CLL patients, especially during those periods of time, when other, less sensitive techniques fail to trace the malignant clone (during chemoimmunotherapy, after stem cell transplant). *Conclusions.* LNA-modified probes and Real-Time PCR technology represent a highly versatile, specific and extremely sensitive methodology for the monitoring of MRD in chronic lymphocytic leukemia. We strongly advocate their usage in the molecular follow-up of MRD in the setting of CLL (and possibly other B-cell malignancies with hypermutated VH gene sequences as well). CLL and related disorders - Clinical / Experimental I

0272

ADOPTIVE IMMUNOTHERAPY OF B-CELL MALIGNANCIES WITH A TRIFUNCTIONAL, BISPECIFIC ANTIBODY (ANTI-CD3 X ANTI-CD20) AND ALLOGENEIC DONOR LYMPHOCYTE TRANSFUSION

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Background. CD20-directed treatment approaches turned out to be highly effective in patients with B cell non-Hodgkin's lymphoma (NHL). But although the chimeric anti-CD20 antibody rituximab induced overall response rates (ORR) of nearly 50% with median response durations of approximately 1 year in relapsed or refractory indolent lymphoma it is not curative and new immunotherapeutic treatment approaches have to be validated. *Aims.* In compassionate use, 3 patients with refractory B-cell chronic lymphocytic leukemia (B-CLL) and 3 patients with refractory high grade non-Hodgkin lymphoma (HG-NHL) were treated with the combination of the trifunctional antibody Bi20 and donor lymphocyte transfusions (DLT). *Method/Study-Design.* The Bi20 antibody is trifunctional, it binds to CD20 and CD3 and activates phagocytosis of the leukemia cell by accessory cells via the Fc part. Bi20 was applied in escalating doses from 10 µg up to 2000 µg and followed by DLT (1×10^7/kg body weight). Patient 2 and 3 received repeated courses of antibody and DLT. *Results.* In 4 out of 6 patients, we observed a prompt, but only transient clinical response. Two patients diseased from HG-NHL did not respond. In cases of B-CLL, a dose-dependent decrease of the leukemic cells was observed even within hours after antibody infusion. Moreover, enlarged lymph nodes and B-symptoms disappeared transiently. Side effects were restricted to fever, chills and bone pain that could be easily controlled. These effects peaked at a concentration of 80 µg and did not increase or even decreased at higher concentrations. The cytokine profile was characterized by a transient increase of IL-6, IL-8 and IL-10. With respect to the transaminases, only a transient and modest increase of γGT was observed. HAMAs (human anti mouse antibodies) were not detectable; their absence allowed repeated application of the trifunctional antibodies. Remarkably, graft-versus-host disease (GvHD) was not observed. Unfortunately relapse of the disease occurred in all cases. In two cases of B-CLL and one case of HG-NHL repeated application of Bi20 and T-cells induced repeated response. *Conclusion.* Bi20 can induce a prompt anti-tumor response in even extensively pretreated patients. The toxicity of treatment is tolerable. However, until now the response is of short duration and further studies are necessary to improve the outcome by e.g. optimizing the application schedule.

0273

TELOMERE LENGTH IS A PROGNOSTIC FACTOR STRONGER THAN VH-MUTATIONAL STATUS IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA (B-CLL)

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Background. Telomere restriction fragments (TRF) length has a prognostic impact in B-CLL. Some studies suggest that this is a mere reflection of its association to VH-mutational status (VH-MS). However, the relative value of these two parameters has not been clearly defined, particularly in cases in which they are discordant. *Aims.* To compare, in a large population of B-CLL patients, the prognostic impact of TRF length and VH-MS, in terms of overall survival (OS), time to first treatment (TTFT) and progression free survival (PFS). PATIENTS AND *Methods.* 184 B-CLL patients have been analyzed for TRF length and VH-MS. All samples were taken before treatment start. Males were 118, females 66. Median age was 62 years (range 34-87). According to Binet staging system, 117 were stage A, 34 B and 33 C. Cytogenetics, CD38 and ZAP-70 expression were available in 80% of patients. Median follow-up was 36 months (range 6-290). Eighty-seven patients have been already treated. TRF length was evaluated by Southern blot and VH-MS by direct sequencing. The standard cut-off of 2% deviation from any germ line VH sequence was employed to define VH-MS. Survival analyses were performed using the Kaplan-Meier method. Cox multiple regression was used to analyze the independence of the following potential prognostic parameters: sex, age, Binet stage, CD38 and ZAP70 expression, cytogenetic features, VH-MS and TRF-length. *Results.* Median TRF length was 6000bp (range 1465-14837bp). There was no correlation between TRF length and patient age, sex or stage. TRF length had a major impact on prognosis with best results observed with a cut-off of 4250bp. Patients with TL<4250bp had a worse outcome than patients with TL>4250bp (median OS: 85 vs 269 months, $p<0.0001$; median TTFT: 21 vs 63 months, $p<0.0001$; median PFS: 12 vs 36 months, $p<0.0001$). VH-MS analysis was successful in 91%. Overall, discordance between VH-MS and TRF length was observed in 16% of patients. Discordance was common among VH-unmutated patients (38%) but rare among VH-mutated patients (6%). Discordant and concordant patients could not be distinguished based on VH usage or degree of homology (H) to the germline IgH sequence (i.e. H=100% vs H<100% and >99% vs H<99% and >98%). In addition they could not be distinguished based on stage, cytogenetics, CD38 and ZAP70 expression. The 24 discordant patients with VH-unmutated status and TRF length>4250bp had a clinical outcome that was significantly different from VH-unmutated patients with TRF length<4250bp (median OS: 83 vs 215 months, $p<0.05$ and median PFS: 12 vs 33 months, $p<0.05$) and similar to that of VH-mutated patients (median OS 269 months and median PFS 54 months, p=n.s.). Finally, the multivariate analysis indicated that TRF length and Binet stage were the most powerful prognostic indicators in B-CLL. *Conclusions.* Our data demonstrate that: 1) TRF length is a major prognostic indicator in B-CLL in terms of OS, TTFT and PFS; 2) when discordance exists between VH-MS and TRF length the latter better predicts outcome.

0274

HIGHLY SENSITIVE DETECTION OF MINIMAL RESIDUAL DISEASE IN B-CELL CHRONIC LYMPHOCYTIC LEUKAEMIA BY INTERPHASE FLUORESCENCE *IN SITU* HYBRIDIZATION ON FLOW SORTED CELLS

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Background. The introduction of new therapeutic agents such as fludarabine and alemtuzumab, with or without autologous or allogeneic stem-cell transplantation, has resulted in increased complete remission rates in B-cell chronic lymphocytic leukaemia (CLL). Preliminary data have suggested that the absence of minimal residual disease (MRD) is an end point of therapy that, if achieved, translates into an improved survival. Future prospective clinical trials that aim toward achieving long-lasting complete remissions should include a test to assess MRD. However, techniques for assessing MRD in CLL show various sensitivity levels and lack standardization. *Aim.*We have developed and validated a combined method to assess MRD in CLL using fluorescence-activating

cell sorting (FACS) and interphase fluorescence *in situ* hybridization (FISH) for the detection of numerical chromosomal aberrations that occur in up to 80% of CLL cases. *Methods.* CLL cells were purified from the peripheral blood of CLL patients by FACS-Aria (BD, US) based on the CD19+CD5+ co-expression, with a purity of > 95%, as assessed by microscopy and by reanalysing with flow cytometry. These CLL cells were shown to harbour either deletion 11q22.3 (ATM) or deletion 13q14 in > 95%, by using dual colour FISH. Peripheral blood samples from normal individuals were spiked with the purified CLL cells with dilutions of 10^{-3} to 10^{-6} white blood cells (WBC). WBC from these spiked samples were subsequently labelled with CD19 and CD5 moAbs and analysed by FACS. $CD19^{+}CD5^{+}$ cell fractions were purified by FACS-Aria and analysed by FISH for either deletion 13q or deletion 11q. *Results.* FISH detection of the specific chromosomal aberration in CD19+CD5+ purified cells allowed discrimination of CLL cells from normal precursor B-cells. Reproducible positive results, above cut-off levels of the probe, were demonstrated in all dilutions up to 10^{-5} or 10^{-6}. Quantification was feasible using the percentage of $CD19^{+}CD5^{+}$ cells and the percentage of aberrant purified cells. *Conclusions.* This approach for the detection and quantification of MRD in CLL reaches a sensitivity at least as high as and even higher than other methods, such as four-color flow cytometry or quantitative allele-specific PCR. It can be used for at least 80% of CLL patients, including all CLL patients with poor prognosis as assessed by the presence of the deletion 11q (ATM) or the deletion 17p (p53). Furthermore, it allows easy standardization among laboratories, applying FACS cell sorting, as it is based on a two-colour labelling only and on FISH assays using commercially available probes. We are now clinically validating the method by assessing MRD levels in intensively treated CLL patients and we propose this method as a candidate approach for assessing the clinical impact of MRD detection in prospective clinical trials on CLL.

0275

MRD KINETIC AFTER AUTOLOGOUS STEM CELL TRANSPLANTATION IN CHRONIC LYMPHOCYTIC LEUKEMIA CAN PREDICT INDIVIDUAL TIME TO RELAPSE AND IS ASSOCIATED WITH CLINICAL OUTCOME AND IGVH MUTATIONAL STATUS

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Introduction. Minimal residual disease (MRD) short after autologous SCT in pts with CLL is known to be close to the detection limit in patients in hematologic remission. Therefore early MRD assessment after SCT is not suitable to predict outcome In patients in remission after SCT. Nevertheless, outcome after SCT is rather heterogeneous even in this population and it has been shown, that the IgH mutational status and other risk factors are of prognostic value after SCT. We therefore analyzed MRD in 61 patients with high risk CLL after myeloablative conditioning regimen of TBI and high-dose cyclophosphamide and consecutive autologous SCT. We established a mathematical model to describe the individual kinetics of MRD-increase after SCT and correlated this to known risk factors as IgVH mutational status, cytogenetics, Lymphocyte doubling time, STK, leukocyte count and clinical outcome. We therefore plotted LOG-MRD levels in each individual patient against time after SCT for an observation period between 12 and 36 months after SCT and calculated patient individual standard curves by linear regression. Significant MRD increase was defined by a change of more than 0.5 orders of magnitude within this observation time, all other cases where regarded as MRD stable or decreasing. 31 of 61 patients showed increasing MRD level with a median slope of 0.08 (0.04-0.88). Assuming that MRD level of 0.5 would be diagnosed as hematologic relapse we could predict the individual clinical relapse by extrapolation with high accuracy (median difference between predicted and observed relapse 1.6 months) in 29 relapsed patients. Patients in ongoing remission showed a significant smaller slope and longer time to predicted relapse compared to relapsed patients (0.09 vs. 0.05 and 48.1 vs. 62.5 months respectively). More important the slope of patients with unmutated VH genes where significant steeper than in mutated cases (0.09 vs. 0.01; p=0.004) whereas other risk factors as LDT, leucocyte count, 11q deletion, STK or status at SCT showed a tendency but not siginificant difference in MRD kinetics. None of these parameters had significant influence on the MRD level within the first year after SCT *Conclusions.* LOG-linear MRD models can characterize CLL increase after SCT: Increasing MRD kinetic predicts the time-point of the clinical relapse with acceptable accuracy in the majority of CLL pts post SCT, whereas decreasing or stable MRD levels are associated with long lasting remission. Absolute MRD levels after SCT are homogeneous low regardless known risk factors, but fast increase of the relapsing CLL clone is correlated to VH unmutated cases. This indicates that the dismal outcome of VH unmutated cases is based on higher proliferating capacity compared to VH mutated cases and not on chemoresistance

0276

MDR-1, BUT NOT MDR-3 GENE EXPRESSION, IS ASSOCIATED WITH UNMUTATED IGVH GENES AND POOR PROGNOSIS CHROMOSOMAL ABERRATIONS IN CHRONIC LYMPHOCYTIC LEUKAEMIA

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Background. The intrinsic or acquired resistance to anticancer drugs remains one of the most significant factors impeding progress in cancer chemotherapy. Although the cellular basis underlying multidrug resistance (MDR) is not fully understood, several factors mediating therapy resistance in tumours have been proposed. One of the mechanisms leading to chemo-resistance in tumour cells is the increased activity by members of the ATP-binding cassette (ABC) superfamily of transport proteins, which function as energy-dependent drug efflux pumps. Two multidrug resistance genes have been identified in humans, MDR-1 and MDR-3. The clinical and biological significance of these MDR mechanisms in chronic lymphocytic leukaemia (CLL) remains to be fully elucidated. *Aims.* This study was designed to investigate whether associations exist between expression of these MDR genes and other markers of poor prognosis, namely unmutated IgVH genes, CD38 expression, adverse cytogenetics and advanced clinical stage in chronic lymphocytic leukaemia (CLL) patients. Additionally, we sought to ascertain whether expression of these genes was dependent on prior exposure to therapeutic drugs. Methods The presence of MDR-1 and MDR-3 was determined using the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique. IgVH mutational status, gene usage, CD38 data, FISH analysis and clinical data were available on all patients. *Results.* One-hundred and one, and 25 patients were tested for the presence of MDR-3 and MDR-1 expression, respectively. Positive and negative controls were included in each batch of APAAP. Twenty-one of 101 patients showed MDR-3 positivity, though no significant associations between MDR-3 gene expression and markers of poor prognosis or exposure to therapeutic agents were evident. MDR-1 expression (19/25) showed a strong associated with unmutated IgVH genes and adverse cytogenetics (p=0.015, p=0.014, respectively). Four patients showed co-expression of MDR-1 and MDR-3, 2 of who have succumbed to their disease. *Conclusions.* In keeping with previous reports, this study demonstrated that expression of both MDR genes is independent of prior exposure to therapeutic agents. Additionally, no associations between advanced clinical stage and expression of MDR genes were evident. MDR expression was, however, associated with shorter survivals than in MDR negative patients. This study highlights the value of determining MDR phenotype in CLL patients, in both refractory and untreated patients. This would allow the design of novel drug regimens containing agents that reverse MDR function, in combination with conventional therapeutic drugs, with the prospect of improving outcomes in CLL.

0277

ANTILEUKEMIC ACTIVITY OF LENALIDOMIDE {REVLIMID} IN PATIENTS WITH RELAPSED OR REFRACTORY CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction. The IMiDs are a new class of immunomodulating agents with antitumor activity against various malignancies. We previously reported the antileukemic activity of thalidomide (T) in combination with fludarabine in CLL pts. Based on this experience we investigated L, a more potent analog of T in pts with rel/ref CLL. Here we report the results of this ongoing phase II clinical trial. *Patients and Methods.* All pts

with rel/ref CLL requiring treatment are eligible. Oral L is given at 25mg/day for 21 out of a 28-day cycle. Treatment is continued until complete response (CR) or progressive disease (PD). NCI-WG 1996 criterion is used to determine response. Three pts had PD and rituximab was added to L. All patients have achieved a PR. (reported separately). *Results.* Nineteen of the 29 pts (median age 64 years; range: 47-75) enrolled are evaluable for response. Duration of monotherapy ranges from 7 to 16 months and combination therapy with Rituximab ranges from 1 to 6 months. Ten pts are inevaluable (3 withdrew consent and 7 received < 2 months of therapy due to toxicity). Major response was noted in 16 of 19 evaluable pts (84%) with 3 CR (2 molecular remission) and 13 PR. All CR's have received monotherapy. *Toxicity.* Most common grade 3/4 adverse effects (AE) were neutropenia (60%) and thrombocytopenia (55%). Another common AE was tumor flare; characterized by tender swelling of lymph nodes and/or rash and low-grade fever, noted in »80% of the pts. One patient developed a DVT and one incurred a PE. *Conclusion.* L is clinically active in CLL pts with rel/ref disease. Hematologic toxicity was the most common AE requiring dose reduction. Overall SE profile was predictable and manageable.

0278

ZAP-70 EXPRESSION IN B-CLL IS ASSOCIATED WITH INCREASED RISK OF AUTOIMMUNE CYTOPENIAS

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Background. autoimmune cytopenias (AIC), namely autoimmune hemolytic anemia (AIHA), thrombocytopenia (AITP), Evans Syndrome (ES) and pure red cell aplasia (PRCA), are relatively common complications B-CLL. The risk of AIC is higher in advanced and heavily treated B-CLL patients. The prognostic impact of AIC on survival is still questioned. *Aim of the study.* To assess the possible correlation of ZAP-70 expression, which is a well-documented prognostic factor in B-CLL, with AIC occurrence. *Methods.* we retrospectively evaluated the incidence of AIHA, AITP, ES and PRCA in 233 B-CLL patients tested for ZAP-70 expression in leukemic cells by immunohistochemistry on bone marrow biopsies (184) and/or flow cytometry on peripheral blood performed within 6 months from diagnosis. *Results.* they were 136 males (58%) and 97 females, aged 34 to 84 years (median 63). At presentation 184 (79%) were Binet stage A, 38 (16,3%) stage B and 11 (4,7%) stage C. Median follow-up was 62 months (range 12 to 387). Overall, AIC was observed in 23 patients (9,8%); 15 were AIHA, 5 AITP, 2 ES and 1 PRCA. In 7 cases (30%) the complication was present at diagnosis (3 AIHA, 2 AITP, 1 ES, 1 PRCA), in the remaining it appeared subsequently, mostly after treatment (alkylating agents in 13, fludarabine in 2). ZAP-70 was expressed in leukemic cells of 18/23 (78%) patients with AIC. The actuarial cumulative incidence of AIC at 10 years was 33±10% in ZAP-70 positive vs 7±3% in ZAP-70 negative cases (p=0.0004). In B-CLL patients developing AIC, survival was lower (24±14% vs 80±4% at 10 years, p=0.0003). Overall survival of all ZAP-70 positive B-CLL patients was significantly shorter (44±9% at 10 years vs 88±4% of ZAP-70 negative cases p=0.00004). ZAP-70 expression was the only significant factor for developing AIC at multivariate analysis (p<0.02). No significant association with age, sex, Binet stage, lymphocyte count or previous B-CLL treatment was found. *Conclusions.* our data suggest that ZAP-70 expression in leukemic cells is independently strongly associated with the risk of autoimmune cytopenias in B-CLL. A possible pathogenetic suggestion might be related to the enhanced signalling via BCR complex induced by ZAP-70.

0279

IN VITRO TREATMENT OF CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) CELLS WITH FLUDARABINE, ETOPOSIDE AND ALEMTUZUMAB (CAMPATH-1H) LEADS TO SPECIFIC MECHANISMS AND RATES OF CELL DEATH IN DIFFERENT GENETIC SUBGROUPS

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Backround. Treatment of CLL with fludarabine, etoposide and the monoclonal anti-CD52 antibody alemtuzumab leads to cell death and clinical responses. The mechanisms by which these processes occur are poorly understood. *Aims and Methods.* In order to gain insight into these mechanisms CLL cells from 41 patients were collected and individually treated with fludarabine (500 µM), etoposide (60 µM) and alemtuzumab (10 mg/ml±cross-linking f(ab')2 fragments) for 24 and 48 hours respectively. Each sample treated with alemtuzumab was also cultured with and without allogeneic serum as a source of complement. In 21 cases T-cell and NK-cell depletion was done using negative selection with anti-CD2 and anti-CD14 magnetic beads. Of 38 cases investigated 25 were VH unmutated and 12 of 39 had del 11q and/or del 17p (n=3). FACS analysis was used to measure rates of cell death with double staining for Annexin V/7AAD and caspase-3 activation. *Results.* Treatment with fludarabine and etoposide induced apoptosis in all but 2 cases, which carried del 17p and del 11q respectively. The rates of apoptosis were lower in cases with genetically high-risk (del 11q or del 17p) CLL, although these cells showed stronger caspase-3 activation than low-risk CLL cells when incubated with fludarabine (see table). Response to alemtuzumab was highly dependent on the presence of serum in the culture: 7% Annexin-V/7AAD-positive cells in serum-free cultures vs 67% in cultures with serum. Addition of f(ab')2 fragments increased the percentage of Annexin-V/7AAD-positive cells even in serum-free cultures. Response to alemtuzumab was independent of the genetic subgroup of the case. Notably, treatment with alemtuzumab in serum containing cultures did not produce cells that stained Annexin-positive/7AAD-negative, a typical feature of early apoptosis, whereas treatment with fludarabine, etoposide and alemtuzumab in serum-free medium resulted in a significant number of Annexin-positive/7AAD-negative cells. This was also observed in T-cell-depleted cultures. In the presence of serum, alemtuzumab did not induce caspase-3 activation, neither did the addition of f(ab')2 fragments. However, in serum-free cell cultures, active caspase-3 was clearly detectable after alemtuzumab treatment, and caspase-3 activity was further up-regulated when f(ab')2 fragments were also added. *Summary.* After *in vitro* treatment of CLL cells with fludarabine, etoposide and alemtuzumab mechanism and rate of cell death differed significantly depending on the genetic subgroup affiliation. CLL cells with high-risk aberrations were more capable of caspase-3 activation when treated with fludarabine or alemtuzumab. Alemtuzumab killed CLL cells independently of serum as a source of complement, but the mechanism of response was different and more effective when serum was added. In serum-free CLL cultures, alemtuzumab induced apoptosis with activation of caspase-3, and addition of cross-linking f(ab')2 fragments increased the rate of apoptosis, whereas in the presence of serum treatment with alemtuzumab induced no typical features of apoptosis, even in T-cell depleted cultures. These findings favor a combination of both CDC and apoptosis but not of ADCC as the cell kill mechanisms activated by *in vivo* alemtuzumab.

Table 1.

	mean% of cells Annexin V+/7AAD+		caspase-3 activation	
	etoposide (48 hrs)	*fludarabine (48 hrs)*	*etoposide (48 hrs)*	*fludarabine (48 hrs)*
/GHₙ unmutated	33%	29%	25%	37%
GHₙ unmutated	58%	35%	24%	14%
del 11q/del 17p	15%	25%	9%	35%
13q/normal karyotype	11%	32%	28%	31%

0280

ANALYSIS OF EXPRESSED AND NON-EXPRESSED IMMUNOGLOBULIN LAMBDA LOCUS REARRANGEMENTS IN CHRONIC LYMPHOCYTIC LEUKEMIA

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In normal individuals, nearly all lambda expressing B cells have rearranged immunoglobulin kappa (IGK) genes and carry IGKV-J junctions, whereas only 2-3% of kappa expressing cells carry IGLV-J junctions. We have recently reported IGK locus rearrangements in the vast majority (97%) of lambda-CLL cases. In the present study, IGL locus rearrangements were analyzed in parallel on cDNA/genomic DNA in 163 kappa- and 104-lambda CLL cases. In all cases, the tumor load was greater than 70%. All experiments were repeated at least three times with identical *Results.* Furthermore, in 156/267 cases repeat samples (obtained at different times) were analyzed and also gave identical *Results.* In lambda-CLL, 110 IGLV-J transcripts were amplified in 104 cases. Two cases carried double in-frame (IF) transcripts: in such cases, the possibility that leukemic cells expressed more than one lambda chain cannot be excluded. Four out of 110 IGLV-J transcripts were out-of-frame (OF); 2/4 OF transcripts were heavily mutated and carried stop codons. DNA-PCR identified additional, non-transcribed IGLV-J rearrangements in 6/104 lambda-CLL cases, of which only one was in-frame. The most frequent genes in transcribed, in-frame rearrangements were IGLV3-21/IGLV2-8/IGLV2-14/IGLV3-1/IGLV1-40. LCDR3 median length was 11 amino acids (range, 8-13); N nucleotides were detected in 50/106 (47,2%) IGLV-J joints; 84/106 cases (79,2%) used the IGLJ2/3 genes, whereas the remainder (22/106; 20,8%) used the IGLJ1 gene. Non-transcribed and out-of-frame rearrangements utilized 9 different IGLV genes and had a median LCDR3 length of 11 amino acids (range, 10-13). N nucleotides were detected in 5/10 IGLV-J joints; 8/10 cases (80%) used the IGLJ2/3 genes. In kappa-CLL, IGLV-J rearrangements were amplified in 10/163 patients (6.1%); 6/10 rearrangements were in-frame. Eight different IGLV genes were identified. Somatic mutations were introduced in 8/10 IGLV sequences. Four out of ten IGLV-J rearrangements in kappa-CLL were also transcribed; 3/4 IGLV-J transcripts were in-frame. In the three kappa-CLL cases with transcribed, in-frame IGLV-J rearrangements, flow cytometry and immunohistochemistry demonstrated that monotypic IG expression was still maintained. In particular, malignant B cells were negative for either cytoplasmic or surface lambda light chains, suggesting post-transcriptional regulation of allelic exclusion. IGLV-J rearrangements in kappa-CLL had a median LCDR3 length of 10 amino acids (range, 9-12); N nucleotides were detected in 5/10 IGLV-J joints; 4/10 cases (40%) used the IGLJ2/3 genes, whereas 6/10 cases (60%) used the IGLJ1 gene. In conclusion, biallelic IGL locus rearrangements are infrequently detected in lambda-CLL. A small subset of lambda-CLL patients have cells that may express more than one lambda chain allele, implying that allelic exclusion of light chains is not absolute. IGL locus rearrangements are infrequent in kappa-CLL, suggesting that the light chain rearrangement *hierarchy* in chronic lymphocytic leukemia (CLL) is not inherently different from normal cells. Differences in IGLJ gene usage between kappa vs. lambda CLL indicate negative selection of the IGLJ1 gene in the expressed CLL repertoire.

0281

ACTIVATION-INDUCED CYTIDINE DEAMINASE EXPRESSION IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Mutation analysis of immunoglobulin heavy chain variable region (IGHV) genes has enabled a subdivision of chronic lymphocytic leukemia (CLL) patients in two subsets, with and without somatic mutations, associated, respectively, with an indolent or a more aggressive clinical course. Nevertheless, regardless of IGHV mutation status, recent data suggest that all CLL cells resemble antigen-experienced and activated B cells. Activation-induced cytidine deaminase (AID) plays a key role in somatic hypermutation (SHM) and class switch recombination (CSR). Given that AID is an essential component of the canonical SHM process in healthy B cells, its expression in CLL might potentially be relevant to the disease. In the present study we evaluated AID mRNA expression in CLL and explored possible associations between AID mRNA expression and surface immunoglobulin (sIg) isotype expression, IGHV mutation status and outcome. Our study group included 130 CLL patients; ten healthy individuals served as normal controls. SIg expression was studied by flow cytometry and/or immunohistochemistry: 13/95 analyzed patients (13.7%) expressed sIgG, whereas the remainder (82/95; 86.3%) expressed sIgM/sIgM+sIgD. Clonal IGH rearrangements were amplified by RT-PCR, gel-purified and directly sequenced; sequence data were analyzed using the IMGT database (*http://imgt.cines.fr*). Using the 98% cut-off value for homology to germline, 82/130 patients (63%) carried mutated IGHV genes, whereas 48/130 patients (37%) carried unmutated IGHV genes. AID cDNA sequences were amplified by RT-PCR with primers covering the entire coding region. Sixty-nine out of 130 patients (53%) carried all three alternatively spliced AID transcripts; 21/130 patients (16%) carried one or two out the three splice variants; finally, 40/130 patients (31%) and all ten healthy individuals were negative for either AID transcript. At least one AID mRNA isoform was detected in all IgG-switched cases vs. 57/82 IgM/IgM,D cases (p=0.02). Detection of all three AID mRNA isoforms was observed in 36/48 IGHV-unmutated cases (75%) vs. 33/82 (40.2%) IGHV-mutated cases (p=0.001). Seventy-eight patients were evaluable for disease progression; 58/78 carried mutated IGHV genes, whereas 20/78 carried unmutated IGHV genes. Among 32 patients with progressive disease, 15 were IGHV-unmutated; the difference in disease progression rate by IGHV gene mutation status was statistically significant (p=0.0003). AID mRNA transcripts were detected in 25/32 (78%) patients with disease progression and 28/46 (60.9%) patients with stable disease (p=0.11, not significant). In conclusion, AID mRNA expression is more frequent in CLL patients with unmutated IGHV genes. IGHV-unmutated CLL cases positive for AID mRNA may be considered to originate from antigen-experienced B cells with inactivated SHM processes or under pressure to maintain their B cell receptor in the unmutated state.

Hodgkin Lymphoma - Clinical trials

0282

RESULTS OF ABVD AND HIGHLY ACTIVE ANTIRETROVIRAL THERAPY (HAART) IN ADVANCED STAGE, HIV-RELATED HODGKINS LYMPHOMA (HL)

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Background and objective. In the HAART era, the results of therapy of HIV-related lymphomas are similar to those observed in non-immumosuppressed patients. There is scarce information on the results of therapy of HIV-related HL using standard chemotherapy together with HAART. The aim of this study was to analyze the results of ABVD regimen + HAART in a multicenter series of 62 Spanish patients with HIV-related HL in advanced stages. *Patients and Methods.* From 1996 to 2005, 62 HIV-infected pts with newly diagnosed HL were treated in 15 Spanish hospitals. HAART was given to all patients from diagnosis if they were not already receiving it. Six to eight cycles of ABVD were planned. G-CSF support was administered according to institutional practices. Response to chemotherapy as well as prognostic factors for response, OS and DFS were recorded. *Results.* Median age 37 yr (range 24-61), 54 (87%) males, 29 (47%) with previous known diagnosis of HIV infection (median from HIV infection diagnosis to HL: 5 yr, range 0-10). Risk activity for HIV infection: IV drug abusers 33 (53%), heterosexual 15 (24%), homosexual /bisexual 13 (21%), unknown 1 (2%). Median CD4 lymphocyte count: 129/mL (range 5-1,209), CD4 count <100/mL: 22 (35%), median HIV load: 1.4x103 copies/mL (range 0-3.9×105), undetectable HIV load: 21/56 (37%). Forty seven (76%) patients were receiving HAART at the time of HL diagnosis (median 13 mo, range 1-109). HL subtype: nodular sclerosis 17 (27%), mixed cellularity 25 (41%), lymphocyte depletion 10 (16%), non-specified HL 10 (16%)(the main reason was diagnosis in extranodal areas). ECOG score ≥2: 22/53 (42%), B symptoms: 55 (89%), stage III: 21(34%) stage IV: 41 (66%), BM involvement: 33/60 (55%). Treatment with the scheduled 6-8 ABVD cycles was completed in 81% of cases. Induction death: 5 pts (8%), CR: 54 (87%), resistance 3 (5%). After a median follow-up of 44 mo, 5 pts have relapsed, with a DFS probability at 5 yr of 74% (95%CI 46-100), and 15 patients have died, being 5-yr OS probability 76% (95%CI 23-89). Causes of death: lymphoma progression 10, HIV-related 3, traffic accident 1, unknown 1. Virologic response to HAART at 6 months after the completion of treatment was observed in 24/36 (67%) evaluable patients. Only lower number of ABVD cycles than scheduled (<6) was a prognostic factor for CR achievement, DFS and OS (OR: 0.153, 95% CI: 0.051-0.462, p=0.001 for CR, OR: 0.137, 95%CI: 0.019-0.979, p= 0.05 for DFS, and OR: 0.153, 95%CI: 0.051-0.0462, p= 0.001 for OS). *Conclusion.* Patients in advanced stage, HIV-related HL treated with ABVD+HAART have a response rate and survival similar to that of immunocompetent patients. The completion of the scheduled therapy was the only factor influencing response and survival in this series.

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0283

HODGKIN'S LYMPHOMA IN ADOLESCENTS - RESULTS FROM THE GERMAN HODGKIN STUDY GROUP

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Background. Both pediatric and adult patients with Hodgkis lymphoma (HL) are commonly treated in seperated treatment protocols. Adolescents are treated with either pediatric or adult protocols depending on study group policies and local legislation. Between 1988 and 1998, the German Hodgkin Study Group (GHSG) did include younger patients from age of 15, and 16, in identically therapy regimens with adult Hodgkin´s lymphoma patients. *Aims.* With a focus on treatment outcome and the recording of secondary malignancies, this analysis aimed to demonstrate wether adolescent patients with HL represent a patient group distinct from adults, possibly requiring a separated therapy strategy. *Methods.* In two GHSG trial generations (G2, and G3), a total of 573 adolescents (15-21 years) in early, intermediate, and advanced stages HL were compared with 4344 adults (22-65 years) for complete remission rate (CR), 5 years survival rate (SV), 5 years freedom from treatment failure (FFTF), and secondary neoplasias (2nd NPL). *Results.* For both adolescents and adults, treatment outcome showed no differences in all stages in terms of CR, SV, and FFTF. A higher rate of 2nd NPLs in the adults patient cohort was detected consistently for early, intermediate, and advanced stages in both trial generations. However, the absolute number of 2nd NPLs in the adolescent group was genarally low. *Conclusion.* Adolescent and adult patients suffering from Hodgkin´s lymphoma show similar therapy outcome when treated with the same regimens. With respect to the small number of cases, a longer follow up is needed to assess the risk of 2nd NPLs particularly in adolescents. Based on this analysis, adolescents seem not to be a distinct patient group with the need for a treatment strategy apart from adult Hodgkin´s lyphoma patients.

0284

INC-EU PROSPECTIVE OBSERVATIONAL EUROPEAN NEUTROPENIA STUDY: PRELIMINARY HODGKIN AND NON-HODGKIN LYMPHOMA RESULTS

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Background. Chemotherapy of malignant lymphomas is often accompanied by major side effects including grade IV chemotherapy-induced neutropenia (CIN) and febrile neutropenia (FN), which may have life threatening consequences in the short term and affect treatment delivery. *Aims.* To assess the incidence, determinants and impact of CIN and FN in routine practice in Western Europe and, ultimately, to develop multivariate risk models of CIN and FN occurrence. *Methods.* A prospective observational study was conducted in 34 centres spread across 5 European countries (Belgium, France, Germany, Spain and UK).

Table 1. Association of RDI ≤ 90% with CIN/FN

	Hodgkin lymphoma	*Non-Hodgkinlymphoma*	*Combined*
Pts. with RDI ≤ 90 (%)			
- if no events	37.5	40.8	40.0
- if grade IV CIN	45.0	53.1	51.5
- if FN	60.0	70.6	68.9
Relative risk (95%-CI) of RDI ≤ 90 (%), compared to pts. with no events			
- if grade IV CIN	1.4 (0.8-2.4)	1.4 (1.1-1.8)	1.4 (1.1-1.7)
- if FN	1.9 (0.6-6.2)	2.3 (1.3-3.9)	2.2 (1.4-3.6)

A total of 307 lymphoma patients were enrolled and observed during their chemotherapy treatment. Treatment was as per usual clinical practice and not influenced by the protocol, except for one blood count at cycle 1 neutrophil nadir. *Results.* Sixty-five patients (21%) were diagnosed with Hodgkin lymphoma (HL) and the remaining 242 (79%) with non-Hodgkin lymphoma (NHL, 66% large B-Cell; 15% follicular; 19% other). Mean age at diagnosis±SD was 39±17 years for HL and 63±13 years for NHL. Men accounted for 56% of the sample in both groups. Ann Arbour stages were distributed I 9%; II 52%; III 19%; IV 20%, and 55% had B symptoms, in the HL group. In the NHL group, stages were distributed I 18%; II 26%; III 16%; IV 40%, and 47% had B symptoms. Chemotherapy regimens for HL patients were ABVD-like (81%), BEACOPP-like (14%) and Stanford V (5%). The regimens used for NHL patients were mainly three-weekly CHOP-like (71%), followed by two-weekly CHOP-like (17%), ACVBP-like (3%) and DHAP/ESHAP-like (3%). Primary prophylaxis with colony-stimulating factors (CSFs) was used in 15% of HL and 26% of NHL patients. Secondary prophylaxis with CSFs occurred in 38% and 28%, respectively. CIN was observed in 46% (95%-CI 34-59%) of HL and 56% (CI 50-62%) of NHL patients. CIN occurrence by regimen type was ABVD-like 39%; BEACOPP-like 78%; Stanford V 33%; three-weekly CHOP-like 22%; two-weekly CHOP-like 55%; ACVBP-like 100%; and DHAP/ESHAP-like 67%. FN occurred in 15% (CI 8-26%) of HL and 21% (CI 16-27%) of NHL patients. Dose delays of ≥ 4 days were observed in 60% of HL patients and 49% of NHL patients. Dose reductions of ≥ 10% were seen in 51% and 54%, respectively. Mean relative dose intensity (RDI) compared to plan±SD was 89±18% for HL and 86±16% for NHL. Low RDI ≤90% was frequent and associated with CIN and FN occurrence (Table). Neu-

tropenia-related hospitalisations were reported for 14% of HL and 17% of NHL patients. *Summary/Conclusions.* A high proportion of lymphoma patients experience CIN or FN and suffer the consequences of hospitalisation and impaired chemotherapy delivery, with a potential for short and long-term sequelae. Further analysis of this data will help to identify new, as well as validate existing risk models. Such models will help to target high-risk patients for prophylactic treatment, in order to decrease the incidence of CIN and FN and allow full-dose chemotherapy to be delivered on schedule.

0285

IFOSFAMIDE, VP16 AND OXALIPLATINE (IVOX) CHEMOTHERAPY FOR PATIENTS IN FIRST PROGRESSION OF HODGKIN LYMPHOMA (HL) AFTER ABVD CHEMOTHERAPY, A SINGLE CENTER PROSPECTIVE STUDY OF 21 PATIENTS

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Background. Despite a high cure rate, 10 to 40% of patients with HL may relapse after complete remission and 5% are refractory to ABVD. Many regimens have been used as salvage chemotherapy with response rate ranging from 40 to 80% depending on patients status (relapsing or refractory). Since 1998, MOPP/ABV chemotherapy has been abandoned and most patients are treated with first line ABVD with cumulative dose of doxorubicine often over 300 mg/m^2. *Aims.* for these reasons we developed a second line treatment without doxorubicine including ifosfamide/etoposide and eloxatine (a platinum component without nephrotoxicity) to reduce disease before high-dose therapy (HDT) and ASCT. *Methods.* the IVOX (ifosfamide 1500 mg/m^2 etoposide 150 mg/m^{2}D1D2D3 and eloxatine 130 mg/m^2 D1) was given every 21 days with GCSF day 6 for 6 days. Twenty one patients with progressive HL (median age 29y) have been prospectively treated from 06/03 to 06/05.Caracteristics of patients: initial stage III/IV (n=11) bulky mediastinum (n = 8), all patients had received ABVD or epirubicin in 3 cases (EBVP) with radiotherapy in 10 cases. At progression 5 patients were induction failure and 16 were in unfavourable relapse (mean time to relapse at 8 mo., stage III/IV at relapse 60%). Patients were evaluated after 2 or 3 IVOX with a PET CT and had PBPC collection beforeHDT. *Results.* according to standard staging criteria 7 patients were in CR/CRu and 7 in PR> 50% giving a response rate of 66.6% and according to PET evaluation, 10 patients had a positive PET CT before intensive therapy. The toxicity was low without hospitalisation for febrile neutropenia, no transfusion, no mucositis. 19 patients had a successful PBPC (2 patients were excluded from PBPC collection, one 66 y with refractory disease and one for viral hepatitis). Among the 19 patients planned for HDT, one died with refractory disease and 18 received HDT (Tandem in 9 cases and RIC allogeneic in one case). At the last follow-up 16 patients are in CCR (with negative PET CT), 2 died from HL and 3 are alive with disease. Conclusion IVOX is a very well tolerated chemotherapy regimen but doesn't appear superior to previous published regimens in progressive HL.

0286

LYMPHOCYTE-PREDOMINANT HODGKINS LYMPHOMA IN CHILDREN: THERAPEUTIC ABSTENTION AFTER INITIAL LYMPH NODE RESECTION IN STAGE I PATIENTS. A REPORT FROM THE SOCIETE FRANCAISE DES CANCERS DE LENFANT (SFCE)

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Background. Lymphocyte-predominant Hodgkin's lymphoma (LPHL) is characterized by early stage, indolent course, excellent prognosis but high risk of second tumors in part treatment-related. In order to clarify the treatment strategy, SFCE has reported its initial experience with a wait and see strategy after adenectomy in a limited number of patients (J Clin Oncol Pellegrino *et al.,* 2003). 2.Aims : to further document patients LPHL s evolution when they received no treatment beyond initial adenectomy. *Methods.* From 1990 to December 2005, 59 patients with LPHL confirmed after pathological review were available for the study. Clinical presentation was: 47 male; median age 10 years (4-17); stage I n=45, stage II n=8, stage III n=5, stage IV n=1. Based on physician decision, 22/45 stage I patients received no further treatment after initial surgery (group SA). 23 patients (group CT) received: combined treatment (n=10), involved field radiotherapy alone (n=3) or chemotherapy alone (n=10). None of them received monoclonal anti-CD20 antibody. The 2 groups were comparable for clinical status and follow up. *Results.* 43/45 achieved CR. All patients with residual lymph node relapsed. With a median follow up of 41 months (6-156), overall survival is 100%. Overall DFS stage I patients is 57% ±10, DFS group SA: 52%±14 and DFS group CT: 63%±13 (p=0.2). Only two patients had TEP-FDG for post surgical evaluation. Median relapse time is 11 months (SA group 5 months/CT group 25 months p=0.2). Stage at relapse was SA group: 5/7 in the same node area and 2/7 stage II; CT group: 4/6 stage I, 1/6 stage III and 1/6 stage IV. *Conclusions.* No further therapy after complete lymph node resection is a valid approach in LPHL comparable to more aggressive approaches. Nevertheless, as most of the relapses involve the same site than the diagnostic a better evaluation of the quality of remission after surgery is to be recommending with TEP and CT/MRI. This could help for an adapted therapeutic approach.

0287

ADMINISTRATION OF FULL DOSE DOXORUBICIN, BLEOMYCIN, VINBLASTINE, DACARBAZINE CHEMOTHERAPY IRRESPECTIVE OF GRANULOCYTE COUNT IN PATIENTS WITH HODGKIN LYMPHOMA: MAINTENANCE OF DOSE INTENSITY WITHOUT GROWTH FACTORS

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Background. Review of existing trials revealed that in most cases administration of ABVD for Hodgkin Lymphoma is subject to dose modifications and the use of growth factors to avoid treatment delays and minimise neutropenia. *Aim.*To investigate whether administration of ABVD irrespective of the granulocyte count causes treatment delays or increases the number of infective episodes in patients with HL. *Methods.* All patients had confirmed HL and were treated with ABVD outside clinical trial protocols, either because they were not deemed eligible, or they declined to take part. Consecutive cases were reviewed for a 5 year period and all were treated on an outpatient basis. *Results.* Thirty-eight patients were treated with ABVD. Median age was 34 (17-68) with 19 males and 19 females. Thirty patients (78.9%) had nodular sclerosis histology, 7 (18.4%) mixed cellularity, and 1 (2.6%) patient had nodular lymphocyte-predominant HD. Twelve patients had stage I (31.57%), 21 (55.2%) had stage II, 2 (5.2%) had stage III and 3 (7.9%) had stage IV disease. Twenty-five patients (65.8%) received 3-4 cycles of ABVD for early HL, while 13 (34.2%) received 6 cycles for advanced disease. The mean number of chemotherapy visits per patient was 4.55 and the total number of chemotherapy visits was 346 (173 cycles). Growth factors were not used in any case. There were in total 14 days of treatment delay (0.28%) and 2 episodes of neutropenic pyrexia during the chemotherapy visits (0.57%). Thirty (78.9%) patients had at least one episode of neutropenia (<1.0×10^9/L) during chemotherapy. The mean number of neutropenic episodes per patient was 3 while the mean granulocyte count in neutropenic patients was 0.6×10^9/L. No dose modifications were performed. Three patients had recurrent disease (7.8%), of which one has received high dose therapy with autologous progenitor cell rescue and is disease-free, while two are currently undergoing salvage chemotherapy. *Conclusions.* ABVD administration irrespective of granulocyte counts did not lead to a higher number of infective episodes and allowed the treatment to be given at full dose without delays. There was no need for growth factor support, minimising treatment costs. The use of full dose ABVD irrespective of granulocyte count should be evaluated in future protocols for HD.

0288

THE IMPACT OF THE SOCIOECONOMIC STATUS IN PATIENTS WITH HODGKIN'S DISEASE IN BRAZIL

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Background. Socioeconomic status (SES) is a determinant of clinical outcome in various types of cancer. *Aim.* The aim of this study was to analyse the impact of the socioeconomic status in patients with Hodgkin's disease (HD). *Methods.* From November 2001 to January 2005, 194 consecutive patients were prospectively followed in five institutions (three public and two private) in Rio de Janeiro. Data regarding disease and treatment features were collected, and patients were classified according to the International Prognostic Score (IPS). Each patient answered a questionnaire about their socioeconomic status, including educational level, household income, ownership of household goods (radio, TV, refrigerator, washing machine, VCR/DVD and car), presence of housemaid, and housing features. Most of these items were used to calculate an index of socioeconomic status the 'Criteria for Economic Classification', which has been validated in publicity and political polls in Brazil. Patients were divided in two groups according to their socioeconomic status: higher SES (classes A1 to C) and lower SES (classes D and E). The IPS score risk was also categorized in low risk (2 or less risk factors) or high risk (more than 2 risk factors). *Results.* There were 151 patients (78%) with a higher SES and 43 patients (22%) with a lower SES. The overall CR rate was 82%, and it was higher in patients with a low risk IPS (87% vs. 72%, p=0.04). Patients with a higher SES had a higher CR rate than those with a lower SES (85% versus 72%, p=0.066, 95% CI of difference:0.45% to 26.22%). The median albumin level at diagnosis was lower in the lower SES group (3.55 versus 3.9, p=0.057) and median age was higher in the lower SES group (34 versus 29, p=0.018).There were no statistically significant associations between the SES group and other relevant variables, including stage, bulky disease performance status, and time from the beginning of symptoms to diagnosis. Ten patients (5%) died during treatment. The causes of death were infection in 9 patients, concomitant advanced disease in 3, and cachexia in one patient. Death during treatment was associated with a lower SES (16% vs. 2%, p=0.001), a lower performance status (p<0.0001), a lower lymphocyte count (p= 0.012), and weakly with a lower albumin level (p=0.065). With a median follow-up of 1.7 years (0.07-4.35), a higher SES was associated with a better 2-year overall survival (93% versus 79%, p=0.01). *Summary/Conclusion.* Lower socioeconomic status was associated with an increased rate of fatal events during treatment, and with a trend towards a lower complete remission rate. Overall survival was lower in the socially deprived patients, apparently due to the higher fatality rate during treatment. Factors indicative of a poor health status at the time of diagnosis appear to explain the observed differences in outcome. In underpriviledged countries, patients with a lower socioeconomic status require a more careful monitoring during treatment, possibly with specific support measures. Regimens more intense then ABVD could pose a prohibitive risk of complications in this group of patients.

0289

POLYMORPHISMS OF GLUTATHIONE S-TRANSFERASE MU1 AND TETHA 1 GENES IN HODGKINS LYMPHOMA RISK

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Background. Hodgkin's lymphoma (HL) is a heterogeneous malignancy, and little is known about the aetiology of this disease. The environmental exposure to cytotoxic and genotoxic agents may be associated with an increased risk of HL. The ability to metabolise carcinogens is variable in human beings. The enzymes of the glutathione S-transferase (GST) system catalyse the conjugation of electrophilic molecules of numerous carcinogenic chemicals, such as benzene and polycyclic aromatic hydrocarbons, to glutathione reducing them to less toxic levels. Genes coding for GST mu1 (GSTM1) and theta1 (GSTT1) proteins are polymorphic in humans and are absent or homozygous null, in 10-60 percent of different ethnic populations. Differences in carcinogen metabolism may explain differences in cancer susceptibility. The association of the GST null genotype and the risk of developing HL are not yet fully clarified. *Aims.* We have tested whether null genotypes for GSTM1 and GSTT1 genes altered the risk for the disease in Brazilian. *Methods.* For this purpose, genomic DNA from peripheral blood of 79 HL patients (40 male, 39 female; mean age±SD: 32.2±14.9 years) and peripheral blood of 367 controls (198 male, 169 female; mean age±SD: 53±4.64 years) was extracted using proteinase K and lithium chloride protocol. GSTM1 and GSTT1 gene were amplified by polymerase chain reaction (multiplex PCR) in the same reaction, including the amplification of a _-globin gene fragment used as a control of the DNA sample. Statistical significance of the differences between groups was calculated by chi-square or Fischer exact test. Crude odds ratios (ORs) were calculated and were given within 95% confidence intervals (CI). *Results.* We have observed similar frequencies of GSTM1 (49.4%) and GSTT1 (17.7%) null genotypes in HL patients and controls (42.5% and 18.0%, respectively; p= 0.32 and p= 1.00). No significant difference was also found in the GSTM1 and GSTT1 null combined genotype frequencies in either group (7.9% vs 11.4%; p= 0.35). Our observation of a 1.32-fold (95%CI: 0.81-2.15) and 0.98-fold (95%CI: 0.52-1.86) risk associated with GSTM1 and GSTT1 null genotypes, respectively, and a 1.54-fold (95%CI: 0.67-3.54) risk associated with the combined null genotype. *Conclusions.* These results suggest that the inherited absence of this carcinogen detoxification pathway may be unimportant determinant of the HL, but a larger number of patients from distinct populations should be analysed to clarify this issue.

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0290

LACE (LOMUSTINE, ARA-C, CYCLOPHOSPHAMIDE, ETOPOSIDE) CONDITIONED AUTOLOGOUS STEM CELL TRANSPLANTATION FOR RELAPSED OR REFRACTORY HODGKIN LYMPHOMA: TREATMENT OUTCOME AND RISK FACTOR ANALYSIS IN 67 PATIENTS FROM A SINGLE CENTRE

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Background. High-dose chemotherapy followed by autologous stem cell transplantation (ASCT) is a recognised treatment option for patients (pts) with relapsed Hodgkin lymphoma. *Patients and Methods.* We analysed 67 pts (46m, 21f, median age at diagnosis 29y, range15-67) who underwent autologous stem cell transplantation (ASCT) after LACE [lomustine (CCNU), cytarabine (Ara-C), cyclophosphamide, etoposide] conditioning for relapsed (n=61) or primary refractory (n=6) Hodgkin lymphoma. The predominant diagnostic histology was nodular sclerosis (n=42), whilst disease stage was I in 2pts, II in 29pts, III in 22pts and IV in 14pts. Median age at ASCT was 32 y (range: 17-70). Prior to ASCT, 40 pts were in complete or partial remission, but 27 pts had less than partial response. *Results.* The 100 day treatment-related mortality was 3%. With a median follow-up of 43.3 months (range 0.5-145.5) the probabilities of overall survival (OS) and progression-free survival (PFS) at 5 years for all 67 patients were 72% and 61%, respectively. Probabilities for OS and PFS at 5 years for patients with chemo-sensitive relapse (n=40) were 79% and 76%, versus 42% and 39% respectively for patients (n=27) with chemo-resistant relapse (p=0.056 for OS, p=0.005 for PFS). In univariate analysis gender, age at diagnosis or at ASCT, extranodal disease, bulk or bone marrow involvement at diagnosis, initial treatment type, response to first line chemotherapy, duration of first remission, time from diagnosis to ASCT, number of treatment lines before ASCT did not have an impact on OS.Three risk factors for worse OS were identified in multivariate analysis (mixed cellularity or lymphocyte-depleted histology, stage III or IV disease at diagnosis, and haemoglobin ≤ 10g/dL at ASCT). Patients with 0 (n=12), 1 (n=26), or 2-3 (n=17) of these three risk factors had 5-year OS probabilities of 100%, 75% and 32% respectively. *Conclusions.* We conclude that LACE followed by ASCT is an effective treatment for the majority of patients with chemo-sensitive relapsed Hodgkin lymphoma. A proportion of apparently chemo-refractory patients may also benefit.

0291

67GA-SPET HAS A ROLE IN PREDICTING DISEASE FREE SURVIVAL (DFS) AND OVERALL SURVIVAL (OS) IN PATIENTS WITH PRIMARY MEDIASTINAL LYMPHOMA AND HODGKIN LYMPHOMA WITH MEDIASTINAL INVOLVEMENT

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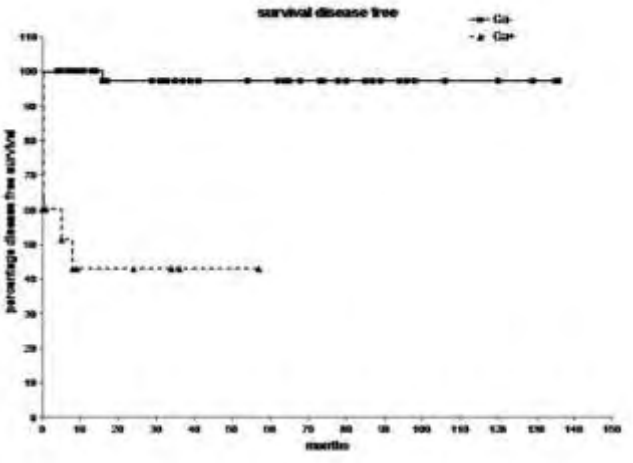
Background. FDG-PET has a superior accuracy than gallium scan (Ga-SPET) in staging and post-therapy restaging of malignant lymphomas. However, in Hodgkin lymphoma (HL) with predominant mediastinal involvement and in primary mediastinal lymphoma (ML), this latter, less expensive, nuclear imaging technique might still have a clinical utility. Aims. The main objective of this prospective study was to assess the predictive value of Ga-SPET in terms of DFS and OS. *Methods.* Ga-SPET was performed 72 hours after intravenous injection of 370 MBq (8-10

mCi) of 67Ga citrate. SPET data acquisition included a 360° rotation, with 60 projections at a rate of 20s per projection. The matrix size was 64x64 and a Butterworth filter (0.4-0.6) was used. Survival curves were calculated by Kaplan-Meyer survival analysis and the comparison between groups was performed by the log-rank test.



Results. The actual final analysis includes 66 evaluable patients (mean age 28, range 12-80) of the 68 initially enrolled in this prospective study. The main disease features of the 66 (43/23 F/M) patients are: stage II/39, III/10, IV/17; histology HL/58 (SN/42, CM/11, DL/1, LP/1, unclassified/3) and ML/8; bulky mediastinal disease yes/no 29/37; B symptoms yes/no, 41/25. Forty-two patients received conventional chemotherapy, 4 non-myeloablative and 20 myeloablative chemotherapy with peripheral blood stem cell support because of unfavourable disease or resistant or relapsing disease to primary treatment. Two patients were excluded because they did not have Ga-SPET at the end of treatment. A total of 109 Ga-SPET/CT restaging were obtained after chemotherapy and/or chemo-radiotherapy completion. Sensitivity, specificity and accuracy were 89%, 91% and 91%, respectively for the Ga-SPET, while they were 100%, 27% and 37% for the CT scan. After a median follow-up of 34 (4-141) months, DFS and OS for patients with a pathological Ga-uptake (Ga-SPET) at the end of the treatment program were 9,5 (2-60) and 26 (9-70) months, respectively. In contrast, the corresponding figures have not been reached for patients with a Ga-SPET indicative of complete remission after a median time of 37+ (3-139) months and 55+ (4-147) months respectively. DFS and OS differed significantly ($p<0.0001$) in patients with pathological Ga-uptake after treatment as compared with patients with a negative Ga-SPET. In contrast, DFS and OS were not significantly different between patients with post-therapy CT scans suggestive of persistent disease and patients with CT scans indicating a complete disease remission (Figure). *Conclusions.* Ga-SPET is still a useful, sensitive and not expensive method to determine the presence of eventual post-therapy active disease in the mediastinum.

0292

AUTOLOGOUS STEM CELL TRANSPLANTATION FOR PATIENTS WITH REFRACTORY OR RELAPSED HODGKIN LYMPHOMA: CLINICAL OUTCOME OF 61 PATIENTS FROM A SINGLE INSTITUTION

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Background. Patients with Hodgkin lymphoma (HL) who do not achieve complete remission (CR) with conventional chemotherapy have poor prognosis. The treatment of choice for these patients is high-dose chemotherapy and autologous stem cell transplantation (ASCT) that may result in prolonged progression-free survival as shown in many studies, particularly registry-based. *Aim.* To investigate the results of ASCT in patients from a single institution with refractory or relapsed HL at the time of the procedure. *Patients and Methods.* Sixty-one patients, 27 males and 34 females with a median age of 31 years (range15-60) transplanted from 1988 to 2005 were analysed. All patients had active HL at the time of ASCT: 31 patients were in partial remission (50%), 18 had refractory disease (30%), and 12 had a non-treated relapse (20%). At transplantation, 26 patients (43%) had advance stage, 15 (25%) presented with B symptoms and 8 (13%) with bulky disease. Seventy percent of the patients had received two or more lines of therapy before ASCT. *Results.* Nine patients (15%) died during the first 3 months after ASCT due to transplant related mortality (TRM). Median follow-up of the surviving patients was 44 months (range 4-140). After haematological recovery, 16 patients received complementary radiotherapy on residual masses. Seventeen patients (28%) achieved CR. Actuarial 5-year overall survival (OS) and disease free survival (DFS) were 50% [95% confidence interval (CI) 42% to 58%] and 47% (95% CI 41% to 53%) for all patients, respectively. Male gender, bulky disease, extranodal involvement, refractory HL, stage IV, B symptoms, and low serum albumin at transplantation were adverse prognostic factors for OS and DFS. In the multivariate analysis, B symptoms and low serum albumin at ASCT were the only adverse prognostic factors significantly influencing OS [relative risk (RR) 4.5, 95% CI 1.6-12.8, $p=0.005$; and RR 3.1, 95% CI 1-9.7, $p=0.05$, respectively]. Bulky disease (RR 6.1, 95% CI 1.9-18.7, $p=0.002$) and low serum albumin (RR 7.3, 95% CI 2.2-24.1, $p=0.001$) adversely influenced DFS. Patients with none of these risk factors had a 5-year DFS of 57% compared to 0% for those with all of these 2 unfavourable prognostic factors. *Conclusions.* Long-term outcome of patients with active HL at the time of ASCT is poor due to a high TRM and a low CR after transplantation. Nevertheless, a subgroup of patients with no adverse prognostic factors at ASCT gains benefit from this treatment.

0293

COMPARISON BETWEEN FLUORODEOXYGLUCOSE POSITRON EMISSION TOMOGRAPHY AND STANDARD RESTAGING IN AGGRESSIVE LYMPHOMA PATIENTS TREATED WITH HIGH DOSE CHEMOTHERAPY AND AUTOLOGOUS STEM CELL TRANPLANTATION

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Background. Positron emission tomography (PET) using (18) Fluorodeoxyglucose (18-FDG) is an important non invasive technique to assess response in lymphoma patients. Early response evaluation by 18-FDG-PET after few cycles of chemotherapy may predict chemosensitivity and then response, progression-free survival and overall survival in this subset of patients. High dose chemotherapy (HDC) followed by autologous stem cells transplantation (ASCT) is reserved to aggressive disease or refractory-relapse disease after standard first line treatment. Chemosensitivity, remission duration and IPI at relapse are best prognostic factors to predict favourable outcome post HDC+ASCT. Early evaluation based on 18-FDG-PET response may be a surrogate of a chemosensitivity test. In this study we compared the use of 18-FDG-PET staging/restaging with standard staging/restaging before and after ASCT in these patients. Aims and *Methods.* From February 2004 to February 2006, 21 aggressive NHL or HD patients with a planned HDC+ASCT were included: 15 males and 6 females respectively with median age of 38 yrs (range 19-63). We included 7 cases of aggressive NHL (5 DLCL and 2 mantle cell NHL at first diagnosis) and 14 cases of HD in relapse or refractory disease. All patients were referred to our Hematology Department for clinical management and were previously studied with conventional staging techniques: physical examination and contrast-enhanced CT of the neck, chest, abdomen and pelvis. Full laboratory tests were performed as well as bilateral posterior iliac crest biopsy for bone marrow evaluation. If necessary, NMR was planned. Before and after ASCT all patients underwent to conventional and 18-FDG-PET restaging. *Results.* Before the ASCT phase the comparison between standard and 18-FDG-PET restaging tests was as follow: CR in 18 pts vs 16 pts and PR in 3 pts vs 5 pts respectively. At that time the restaging by conventional procedure and 18-FDG-PET was concordant in 17 pts: 15 pts in CR and 2 pts in PR respectively. Discordant restaging was observed only in 4 pts. The final evaluation was concordant with both procedures in 20/21 pts: we observed 17 CR and 3 PD. One patient was in PR at the 18-FDG-PET and in CR at the standard evaluation. Involved field radiotherapy was added in the 5 pts with discordant restaging before or after ASCT with achievement of CR. At a median FU of 18 months only 1/17 pts who achieved CR at the end of treatment was in PD. *Conclusions.* 18-FDG-PET is an important imaging technique for the end-treatment evaluation in lymphoma disease, because it may better define CR patients. However, 18-FDG-PET findings must be correlated with clinical data, others imaging analysis and, if necessary, bioptic specimens. Indeed, more large studies are needed to determine the real impact of 18-FDG-PET on early evaluation before ASCT in aggressive NHL or HD patients.

Hodgkin Lymphoma - Clinical trials

0294

CLINICAL RESISTANCE TO PRETRANSPLANT IMATINIB THERAPY IS AN ADVERSE PROGNOSTIC FACTOR FOR THE OUTCOME OF ALLOGENEIC STEM CELL TRANSPLANTATION IN CML

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Background. The ABL tyrosine kinase inhibitor imatinib is highly effective in the treatment of chronic myelogenous leukemia (CML). However, due to short follow up the long term effects of imatinib are unknown. At present, allogeneic transplantation remains the only treatment with curative potential. *Aims.* In order to determine the effect of imatinib therapy before allogeneic transplantation we analyzed patients who had received imatinib as part of pretransplant therapy with respect to clinical response or resistance to imatinib therapy. *Patients and Methods.* Clinical resistance to imatinib was defined as 1) primary cytogenetic unresponsiveness or 2) detection of increasing percentage of Ph+ metaphases and/or increasing BCR-ABL positive interphase nuclei in FISH analysis, and 3) hematological progress under ongoing imatinib therapy. Fifty eight patients from two centers were evaluable. The median age was 46 years (range 17-65). Twenty patients were transplanted in 1st chronic phase (CP), 38 in 2nd and higher CP. Seventeen patients had a sibling donor, 41 had an unrelated donor. The median follow up time after allogeneic transplantation was 360 days (range 24-1524). *Results.* Imatinib resistance, stage of disease, time from diagnosis to transplantation, and age were significant prognostic factors for overall survival (OS: p<0.001, p=0.001, p=0.027, p=0.016, respectively) and leukemia free survival (LFS: p<0.001, p=0.002, p=0.058, p=0.042, respectively) in univariate analysis. Multivariate analysis by Cox regression demonstrated that clinical resistance to imatinib was an independent adverse risk factor for OS (p=0.006) and LFS (p<0.001). Stage was the only other independent risk factor for LFS (p=0.045) and OS (p=0.057). *Conclusion.* Our data suggest that allogeneic HSCT should be planned as long as there is cytogenetic response to imatinib.

0295

IL-12, MYELOID DENDRITIC CELLS AND THE TH1 MODEL IN ACUTE GRAFT-VERSUS-HOST DISEASE AFTER REDUCED INTENSITY CONDITIONING ALLOGENEIC STEM CELL TRANSPLANTATION

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Inflammatory cytokines act as mediators of aGVHD. The use of RIC regimens has modified the natural history of transplant-related complications, especially aGVHD. Our current knowledge of the pathophysiology of aGVHD is based primarily on results obtained in the myeloablative setting. The aim of this study was to investigate the role of inflammatory cytokines on aGVHD incidence and severity in 113 patients who received a RIC allo-SCT from an HLA-identical sibling. Plasma levels of: IL-1β, IL-6, IL-8, IL-10, IL-12p70, IL-18, TNF-α, IFN-α, IFN-γ, and Fas-ligand, were measured by ELISA prior to allo-SCT, at day 0 prior to graft infusion, and at regular times within the first 3 months after allo-SCT. Except for IL-12p70, all measured cytokines showed little variations in the first three months. The incidence of grade II-IV aGVHD was 45% (95%CI, 36-54%; median onset, 32 days after allo-SCT). In the subgroup of patients for whom all tested cytokines could be measured closely, but rigorously prior to aGVHD clinical onset, a high IL-12p70 level (p<10-4) measured around the first month after allo-SCT were significantly associated with the development of clinically significant grade II-IV aGVHD. IL-12p70 levels were significantly correlated to the severity of aGVHD: grade 0-I, median 468 pg/mL; grade II, median 2538 pg/ml; and grade III-IV, median 4615 pg/mL (p<.0001). In patients experiencing grade II-IV aGVHD, IL-12p70 levels decreased after aGVHD therapy. Interestingly, we found a more rapid recovery of monocytes, that are the main pool of IL-12p70-secreting myeloid dendritic cells (DC), prior to aGVHD clinical onset in patients with grade II-IV aGVHD, as compared to patients with grade 0-I aGVHD (median, 829/L vs. 552/L; p=.005). At the effector level, we observed a significantly more robust recovery of genuine naive CD3+CD4+CD45RA+CD27+ T cells prior to aGVHD clinical onset, in patients with grade II-IV aGVHD, as compared to patients with grade 0-I aGVHD (median, 50/L vs. 16/L; p=.006). Finally, in multivariate analysis, IL-12p70 level measured before aGVHD clinical onset was the strongest predictive factor for aGVHD development and severity (p<10-4; RR=10.7; 95%CI, 3.8-30.6). Overall, these findings reconstitute a genuine Th1 loop, supporting a model where aGVHD primarily reflects a type 1 alloreaction (rapid monocytes/DC recovery, IL-12p70 secretion, naive CD4+ T cells expansion, Th1 and Tc1 cells differentiation) in the context of RIC allo-SCT.

0296

MOLECULAR REMISSION IN FOLLICULAR LYMPHOMA AND CHRONIC LYMPHOCYTIC LEUKEMIA AFTER REDUCED INTENSITY ALLOGENEIC STEM CELL TRANSPLANTATION CORRELATES WITH A BETTER DISEASE FREE SURVIVAL

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Background. Reduced intensity allogeneic stem cell transplantation (RIC allo-SCT) can be an effective salvage treatment for relapsed chronic lymphocytic leukemia (CLL) and follicular lymphoma (FCL). In our series, progression-free survival is 80% and 60% at 4 years for FCL and CLL patients (pts) respectively. In the autologous SCT setting, it has been shown that the attainment of clinical and molecular remissions can be predictive of a better disease free survival (DFS). *Aims.* Aim of this study was to investigate whether RIC allo-SCT is able to induce durable clinical and molecular remissions (MR) in relapsed FCL and CLL pts and whether minimal residual disease (MRD) status correlates with a better survival. *Methods.* Thirty-four pts (16 CLL and 18 FCL), having a molecular marker, were in complete remission (CR) after a RIC transplant (containing thiotepa, fludarabine and cyclophosphamide) from a HLA-identical sibling (n=32), unrelated (n=1) or haploidentical (n=1) donor. The median age was 52 years (range, 32-69 years). The median number of previous treatments was 2 (range, 1-5); 23% pts had failed a previous autologous SCT. Before transplant, 11 pts (32%) were in CR, 14 pts (41%) were in partial remission and 9 pts (27%) were in progressive or stable disease. Bcl-2 (n=15) or immunoglobulin heavy chain gene rearrangements (IgH, n=19) were used as molecular markers. After allo-SCT, serial BM samples were analyzed for MRD by nested-PCR. The median molecular follow-up was 24 months (range, 6-64). *Results.* Overall, 24 of 34 pts (71%) pts attained MR, 7 pts (20%) were PCR-positive and 3 pts (9%) showed an intermediate pattern of PCR positivity and negativity. All but one of the PCR-negative pts achieved MR within the first year after allo-SCT. Sixteen of 18 FCL pts (89%) achieved MR, while only 8 of 16 CLL pts (50%) were MRD-negative at the last follow-up (p=0.002). FCL and CLL pts were not different for number of previous treatments, pre-transplant disease status and incidence of chronic and acute graft versus host disease (GVHD). Among pts who were persistently PCR-negative, only one CLL pts relapsed at a nodal site that showed Richter transformation, while among pts who were PCR-positive 4 pts relapsed. The difference was statistical significant (p= 0.0051) and translated into a better DFS for PCR-negative pts (94% vs 33%, p< 0.0001). None of the pts who relapsed experienced GVHD, while all the pts who were persistently PCR-positive or had an intermittent pattern without relapsing showed acute or/and chronic GVHD. Eighty percent of PCR-negative patients developed GVHD that preceded or was concomitant with the achievement of MR. The overall incidence of chronic GVHD among PCR-negative pts was 54%, and among pts who were PCR-positive or had an intermediate pattern and did not relapse was 67% (p=0.67). *Conclusions.* i) MR can be attained after RIC allo-SCT in the large majority of FCL pts and in 50% of CLL pts; ii) the achievement of MR correlates with a lower relapse risk and a better DFS; iii) MRD monitoring can be used to tailor post-transplant immunotherapy.

0297

IMPACT OF T-CELL CHIMERISM AFTER REDUCED INTENSITY CONDITIONING ALLOGENEIC STEM CELL TRANSPLANTATION

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Here, we investigated the impact of different factors on the establish-

ment of full donor CD3+ T cell chimerism (TCC) in a series of 102 patients receiving RIC allo-SCT from an HLA-identical sibling. 65 patients (64%) received an ATG-based RIC regimen (fludarabine, busulfan and ATG), 14 patients (14%) received a low dose TBI-based RIC (2 Gy total dose), while the remaining 23 patients (23%) received an association of fludarabine, busulfan and total lymphoid irradiation (TLI). At day 30, 30% (95%CI, 21-39%) of patients achieved a full TCC of donor origin in the peripheral blood. At day 90, 77% (95%CI, 69-85%) had a full donor TCC. In univariate analysis, none of the patients', graft, RIC type, or disease characteristics were predictive of establishment of an 'early' full donor TCC at day 30 after allo-SCT. However, the group of 31 patients who achieved a full donor TCC by day 30, had a significantly higher incidence of grade 2-4 acute GVHD, in comparison to the group of 71 patients who were still in mixed TCC at day 30 (cumulative incidence, 61% vs. 35%; p=0.01). When looking for predictive factors for full donor TCC at day 90, univariate analysis showed that diagnosis category, the RIC regimen type (ATG, TBI or TLI-based RIC), a female donor, CD34+ stem cell dose, and CD4+ T cell dose infused, were significantly or had a trend towards significant association with the establishment of full donor TCC by day 90. In the multivariate analysis, a diagnosis other than a myeloid malignancy, was the strongest parameter significantly predictive of establishment of full TCC at day 90 after RIC-allo-SCT (p=0.007; OR=3.82; 95%CI, 1.4-10.1). Most importantly, the delayed establishment of full donor TCC in patients with myeloid malignancies translated towards a worsened PFS (p=0.06) in the group of 15 patients who did not achieve full donor TCC at day 90 as compared to the group of 26 patients who achieved a full donor TCC. This worsened PFS was due to a significantly higher incidence of leukemia relapse among these 15 patients (6 relapses; 40%) as compared to none in the other group of 26 patients (p=0.002). We conclude that cautious monitoring of the levels of donor TCC is mandatory after RIC allo-SCT, because this can improve patients' outcome through identification of patients at risk for acute GVHD, and disease progression, and guidance of early interventions with immunosuppressive drugs or donor lymphocyte infusions aimed at obviating these complications.

0298

B-CELL CONCENTRATION IN THE APHERETIC PRODUCT PREDICTS ACUTE GRAFT-VERSUS-HOST-DISEASE AND TREATMENT-RELATED MORTALITY OF ALLOGENEIC PERIPHERAL BLOOD STEM CELL TRANSPLANTATION

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Background. More than 60% of allogeneic stem cells transplants are at present performed utilizing donor derived peripheral blood stem cells (PBSC) after mobilization with granulocyte-colony stimulating factor. The effects of the cellular composition of the inoculum on the outcome of these patients have so far not been fully dissected. *Aims.* Aim of this study was to correlate the clinical parameters of the post-PBSC transplant procedure - in particular engraftment, acute and chronic graft-versus-host-disease (GVHD), relapse, treatment-related mortality (TRM), overall survival (OS), leukaemia-free survival (LFS) and event-free survival (EFS) - with the cellular composition of the inoculum in terms of concentration of nuclear cells, mononuclear cells, T cells, B cells and NK cells. *Methods.* The analysis has been performed on the apheretic products collected for 63 consecutive patients (27 AML, 10 ALL, 13 CML and 13 MM) who underwent an allogeneic PBSC transplant from an HLA identical sibling between November 1999 and November 2005. The conditioning regimen was represented by Busulphan 16 mg/kg of body weight (BW) plus Cyclophosphamide 120 mg/kg BW (AML, CML and MM patients) or TBI 12 Gy plus Cyclophosphamide 120 mg/kg BW (ALL patients). Cyclosporine plus Methotrexate were given to all patients for GVHD prophylaxis. Hemochromocytometric analysis and three-color immunofluorescence were performed on the apheretic samples, using antibodies against CD3, CD4, CD8, CD20, CD16, CD56 and CD34. Actuarial curves for OS, LFS and EFS have been calculated according to the Kaplan-Meier method; cumulative incidence has been used to evaluate the probability of engraftment, GVHD, relapse and TRM; finally, the correlation with the number of cells and cellular subsets infused has been calculated by means of the correlation coefficient of Spearman. All the variables for which a p-value <0.1 was recorded underwent a multivariate analysis using the proportional model of Cox. *Results.* The median number of infused cells/Kg of the recipient BW was: nucleated cells 10.9×10^8 (range 5-51), mononucleated cells 6.7×10^8 (range 1.56-21); CD34 5.9×10^6 (range 4.07-14); CD3/CD8 0.9×10^8 (range 0.19-3.1); CD3/CD4 1.5×10^8 (range 0.4-8.1); CD3 2.6×10^8 (range 0.07-11.30); CD20 0.28×10^8 (range 0.06-2); CD16/CD56 0.20×10^8 (range 0.01-1.7). Acute GVHD was observed in 24 patients (grade I-IV). In multivariate analysis, acute GVHD correlated with the disease (p 0.002), with the phase of the disease at the time of transplant (p 0.047) and with the number of CD20+ infused cells (p 0.06). Greater the number of CD20+ cells present in the inoculum and higher was the probability of developing acute GVHD. The high number of CD20+ cells in the inoculum also correlated significantly with the TRM (p 0.02). *Summary/Conclusions.* The results of this analysis suggest that the concentration of B cells in the apheretic product may predict the incidence of acute GVHD and TRM in patients undergoing an allogeneic PBSC transplantation, thus influencing the clinical outcome. This is in agreement with the recent evidences regarding the role played by B cells in the pathogenesis of GVHD. These findings suggest possible new preventive and therapeutic strategies in the clinical management of GVHD.

0299

ALLOGENEIC HEMOPOIETIC STEM CELL TRANSPLANTS (HSCT) FOR PATIENTS WITH RELAPSED ACUTE LEUKEMIA : LONG TERM OUTCOME

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Background. Patients with acute leukemia may be referred for allogeneic hemopoietic stem cell transplantation (HSCT) at the time of relapse. The outcome of allogeneic HSCT in these patients is relevant when discussing treatment strategies and donor selection. *Aim of the study*. To assess the long term outcome of 152 patients with acute myeloid (AML) or acute lymphoid leukemia (ALL) undergoing an allogeneic HSCT in our Unit between 1977 and 2004. *Patients.* We have allografted 152 patients with relapsed AML (n=86) or ALL (n=66). The median blast count in the marrow was 30% (7-100), and the median blast count in the peripheral blood was 2 (0-99). Median age was 31 (11-62), and the median year of transplant 1995 . Conditioning regimen included total body irradiation (TBI) (10-12 Gy) in 115 patients. The donor was a matched sibling donor (MSD) in 106 , a family mismatched donor (FMD) (n=20) or an unrelated donor (UD) in 26. The graft was T cell depleted (TCD) in 12 cases. Leukemia was diagnosed in first relapse in 42 and more advanced disease, or primary refractory in 110. *Results.* The overall actuarial survival at 20 years is 15%, the cumulative incidence of transplant mortality (TRM) is 42%, and the CI of relapse related death (RRD) is 43%. There was no impact of stem cell source and no improvement of results with time (</=> 1995). In multivariate analysis on survival favorable predictors were the use of a donor other than family mismatched (RR 0.45); and bone marrow blast count less than 30% (RR 0.82). The actuarial 20 year survival for 65 patients with both favorable predictors is 26%. When the analysis is restricted to 94 patients surviving 100 days, the presence of chronic GvHD was the strongest favorable predictor (RR 0.38, p=0.0008) followed by donor other than family mismatched (RR 0.32, p=0.008), donor age less than 34 years (RR 0.55, p=0.02), and blast count less than 30% (RR 0.58, p=0.07). For 18 patients with all 4 favorable predictors, the actuarial 20 year survival is 54%. *Conclusions.* This study confirms that 15% of relapsed leukemias can be cured with an allogeneic transplant . The use of young, HLA matched donors and a marrow blast count less than 30% significantly increases the likelihood of long term survival , which is further improved if chronic GvHD develops. This may be relevant when discussing transplant strategies in patients with relapsed leukemia.

0300

TREATMENT OF REFRACTORY CHRONIC GRAFT-VERSUS-HOST DISEASE WITH EXTRACORPOREAL PHOTOPHERESIS

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Chronic GvHD is the major late complication of allogeneic bone marrow transplant with significant impact on late mortality. Systemic steroids are the first line therapy with only 30-40% complete responses. ECP has been proposed as an alternative therapy for immune-mediated diseases, including transplant rejection and GvHD. Aim of the study.

This is a single Center study testing the efficacy of ECP in patients with steroid-resistant cGvHD. Patients. Twenty-six patients entered this study. Their median age was 40 (range, 5-61) years. The median interval from diagnosis of cGvHD to ECP treatment was 12 (range, 6-168) months. All patients received at least 2 lines of immunosuppression including cyclosporine (CyA) alone, CyA and steroids, CyA and mycophenolate mofetil (MMF), steroids and MMF, steroids and tacrolimus. Methods. Patients were treated on 2 consecutive days (one cycle) at 1 week interval for the first month, at 2 weeks interval for the second month and at 4 weeks interval for the subsequent 4 months, for a total of 10 cycles. At 6 months a decision was made whether to continue the ECP treatment as monthly maintenance, depending upon the clinical response. During ECP treatment, the patients continued to receive the baseline immunosuppressive therapy and were followed in the outpatient clinic. Results. After a median of 15 (range, 7-33) cycles, 11 (76%) of the 14 patients with skin involvement had a partial or complete response. In particular, of 6 patients with severe scleroderma 2 had complete resolution of skin contraction, abrasion and thickening, 2 showed a significant improvement and 2 had no response. Of 15 patients with gut involvement, 8 showed a complete response and 3 a partial response. A return to normal values or reduction of abnormal liver function enzymes by at least 50% from baseline were observed in 9 of 12 patients (75%) with liver involvement. Of 9 patients with ocular symptoms due to sicca syndrome, 6 had a complete or partial response. A complete response was observed only in 1 of 3 patients with lung cGvHD. The first signs of response appeared at a median of 3 months. Throughout the ECP treatment course systemic immunosuppressive medication was increased in 4 patients, reduced in 8 and discontinued in 14. The steroid therapy was discontinued at a median of 4.5 months. At a median follow-up of 41 (range, 3-60) months, 22 (84%) patients are alive, 3 patients died of cGvHD related infectious complications and 1 of leukemia relapse. Twelve patients (46%) discontinued ECP after a median of 18 (range, 6-24) cycles because of complete and sustained resolution of cGvHD and 5 patients (19%) because of minimal or inadequate response. The ECP treatment is ongoing in 5 patients. The procedures were well tolerated and completed in all cases and no relevant adverse events were observed. Conclusions. Our results confirm the role of ECP in controlling refractory cGvHD. The response of a single organ is independent from the others. The response of liver cGvHD is comparable to mucocutaneous response. New strategies are requested for lung cGvHD.

0301

IMPACT OF DISPARITIES AT SINGLE OR MULTIPLE HLA LOCI ON OUTCOME AFTER UMBILICAL CORD BLOOD TRANSPLANTATION FOR ADULTS WITH HEMATOLOGIC MALIGNANCIES

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Background. The number of HLA disparities considering HLA-A, -B, and 'DRB1 is strongly related to engraftment, disease free survival (DFS), and overall survival (OS) in children undergoing UCBT. The influence of HLA disparities on outcome after UCBT in adults is unknown. Studies on the relevance of HLA mismatching at allele level after UCBT are lacking. *Aims.* To evaluate the possible influence of the number of disparities at single or multiple HLA loci on outcome after UCBT in adults with hematologic malignancies. *Patients and Methods.* The impact of HLA mismatching on myelod engraftment, graft-versus-host disease (GvHD), transplant-related mortality (TRM), relapse risk (RR), DFS, and OS was analyzed in a series of 78 adults with high-risk hematologic malignancies transplanted at our center from May 1997 to September 2005. Median age was 30 years (range, 16-47) and median weight was 70 kg (range, 41-112). Diagnosis were high-risk acute leukemia in 47, chronic myelogenous leukemia in 21, high-risk myelodysplastic syndrome in 5, and others in 5. The status of the disease at transplant was advanced in 25 cases (30%). In the first 72 patients conditioning consisted of thiotepa, busulfan, cyclophosphamide and antithymocyte globulin (ATG). The last 6 patients received thiotepa, fludarabine, busulfan and ATG. All patients received cyclosporine and prednisone for GvHD prophylaxis, and filgrastim to fasten engraftment. HLA individual loci analyzed were HLA-A, -B, -C, -DRB1, -DQB1 and -DPB1. The combinations of loci evaluated were HLA-A + B + DRB1, HLA-A + B + C + DRB1, HLA-A + B + C + DRB1 + DQB1 and HLA-A + B + C + DRB1 + DQB1 + DPB1. All analyses were performed considering the number of mismatches present both at allelic and antigen level and in the HVG direction and in both directions (GVH and HVG). *Results.* The degree of HLA match (HLA-A and -B by low-resolution and -DRB1 by high-resolution DNA typing) was 6/6 in 4 (5%), 5/6 in 27 (35%), and 4/6 in 47 cases (60%). The median number of nucleated cells and CD34+ cells infused was 2.2×10^7/kg (range, 0.9-4.9) and 1.1×10^5/kg (range, 0.1-5.7) respectively. The median time to neutrophils > 0.5×10^9/L was 21 days (range, 11-57). The probability of developing grade II'IV acute GVHD was 39% (grade III-IV, 21%), and of developing chronic GVHD was 54% (extensive, 35%). With a median follow-up of 32 months (range, 3'101), the probability of DFS and RR at 3 years was 36% and 30% respectively. On multivariate analysis, only the presence of mismatch in locus HLA-A by low resolution in direction HVG was related to myelod engraftment. *Conclusions.* The degree of HLA mismatching seems not as important in adults as compared to children undergoing UCBT. These data show the high-resolution DNA typing for HLA-A, -B, -C, -DRB1, -DQB1 and 'DPB1 is not essential in UCB searches for adults.

0302

CORTICOSTEROIDS FOR PREVENTING GRAFT-VERSUS-HOST DISEASE AFTER MYELOABLATIVE STEM CELL TRANSPLANTATION: A COMPREHENSIVE META-ANALYSIS

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Background. Graft-versus-host disease (GvHD) remains a major complication in allogeneic myeloablative stem cell transplantation (SCT) and is considered as the main cause for transplantation related morbidity limiting its wider application. The current standard regimen for preventing GvHD combines cyclosporine A (CSA) with a short-course of methotrexate (MTX). The question if the addition of steroids improves patients' outcomes has not been clarified yet as the results of single studies are ambiguous. *Aims.* To determine the effectiveness of corticosteroids used for the prevention of GvHD after myeloablative SCT in improving overall survival (OS), disease-free survival (DSF), relapse incidence (RI), non-relapse mortality (NRM), acute GvHD grade I-IV, II-IV and III-IV and chronic GvHD. *Methods.* We conducted a comprehensive literature search in Cochrane Library, EMBASE, MEDLINE, internet databases for ongoing trials, and conference proceedings (1975-2004). Randomised controlled trials evaluating GvHD prophylaxis regimens differing only in the use of corticosteroids were included. A minimum of 75% of the patients undergoing allogeneic myeloablative SCT had to be adults. All authors were asked to provide unpublished and/or missing data. Trial selection, quality assessment and data extraction were done independently by two reviewers. To analyse outcomes with time-to-event data hazard ratios (HR) were calculated on the basis of individual patient data or *if not available* extracted from the publication using well-established *Methods.* The weighting was done according to the method of Peto, which assumes a fixed effect model. Heterogeneity of treatment effects between the trials was assessed by using a Chi-squared test with a significance level of $p<0.1$. *Results.* 1,709 references were screened, of which 5 randomised controlled trials with 604 patients were finally included in the review. The addition of corticosteroids reduced statistically significant the incidence of acute GvHD grade I-IV (HR 0.58, 95% CI 0.45 to 0.76) as well as grade II-IV (HR 0.69, 95% CI 051 to 0.92). No significant differences seen for acute GvHD grade III-IV (HR 0.78, 95% CI 0.52 to 1.15) and chronic GvHD (HR 1.21, 95% CI 0.89 to 1.65) as well as no improvements were found for OS (HR 0.99, 95% CI 0.79 to 1.25), DFS (HR 0.95, 95% CI 0.74 to 1.23), RI (HR 0.82, 95% CI 0.57 to 1.18) or NRM (HR 0.88, 95% CI 0.61 to 1.26). *Summary/ Conclusions.* The addition of corticosteroids to GvHD prophylaxis regimens reduces the risk for acute GvHD grade I-IV and II-IV. However, based on the randomised trials currently available there is no evidence that this benefit improves long-term outcomes such as OS, DFS, RI, NRM or chronic GvHD.

0303

REDUCED INTENSITY ALLOGENEIC STEM CELL TRANSPLANTATION IN PATIENTS WITH MYELODYSPLASTIC SYNDROME: DOES THE CONDITIONING MATTER? A RETROSPECTIVE STUDY OF THE SFGM-TC ON 61 PATIENTS

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Reduced-intensity allogeneic stem cell transplantation (RIA) has emerged as an alternative to myeloablative transplantation in patients with myelodysplastic syndrome (MDS). Given the uncertainty regarding the appropriate conditioning, SFGM-TC conducted a retrospective multicenter study with the attempt to evaluate the impact of conditioning on patients' outcome. The record of 61 patients (37 males) with MDS who received a RIA between 1998 and 2003, from 22 French transplantation centres, were reviewed. Participating centres were asked to verify data referred to French registry and provide additional information on each patient. According to the FAB classification, 11 patients had RA at diagnosis, of whom one had progressed to REAB and one to AML before transplantation. Thirty two patients had REAB at diagnosis, of whom 2 had progressed to REAB-T and 7 to AML before transplantation. Twelve patients had REAB-T at diagnosis and 6 CMML, of whom 8 progressed to AML before transplantation. The median time from diagnosis to RIA was 12 months (6-129). Conditioning regimen consisted of Fludarabin (Flu) plus busulfan (FB; n=29), Flu plus 2-Gy TBI (F-TBI; n=20) and idarubicin plus aracytine and Flu (FlagIda; n=12). Donors were HLA-identical siblings (n=52) and HLA-matched unrelated (n=9). All pts received peripheral blood stem cells. The median of CD34+ infused cell dose was 5×10^6/kg (0.5-17.3). At the reference date of analysis of 1 July 2005, median follow-up was 44.7 months (21-85). Estimated 3-year overall survival (OS), progression free survival (PFS), relapse and transplant-relapse mortality (TRM) were 35%, 27%, 66% and 30%, respectively. Neither of the 3 conditioning regimens used (FB, F-TBI and FlagIda) had impact on patients' outcome. In multivariable analyses, while acute III/IV grade GVHD development was the only factor found to adversely influencing OS (HR=3.6; 95% CI: 1.1-12.2), chronic GVHD development was the only favourably influencing PFS and relapse ratios (HR=0.3; 95% CI: 0.1-0.7 and HR=0.2; 95% CI: 0.1-0.6, respectively). TRM was adversely influenced by male sex of patient (HR=9.2; 95% CI: 1.5-66.6). RIA seems to be an effective treatment in MDS patients irrespective of conditioning type. While acute III/IV grade GVHD appeared to be detrimental, the benefit effect of chronic GVHD was to be bound to GVL effect as demonstrated by the improvement of PFS and relapse rates in patients who developed chronic GVHD. New approaches with focus on immunosuppressive treatment are needed to enhance the GVL effect with an acceptable risk of GVHD.

0304

PROGENITOR CELLS ARE TRAPPED IN FILTERS USED FOR MARROW HARVEST: RECOVERING CELLS FROM MARROW FILTERS REDUCES GRAFT VS HOST DISEASE AND TRANSPLANT MORTALITY

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Background. A bone marrow harvest is filtered either in the operating room, in the laboratory, or during infusion to the patient. Filters are usually discarded. Little is known of haemopoietic progenitor cells trapped in the filters. *Aim of the study*. To evaluate haemopoietic progenitor cell content in the filters and to assess the outcome of transplants with filter-discarded or filter-recovered cells. *Patients and Methods.* Haemopoietic progenitors were grown from filters of 19 marrow transplants. We then compared the outcome of 39 filter-recovered transplants from HLA identical siblings (years 2001-2004) with a matched cohort of 43 filter-discarded marrow grafts (years 1997-2000). *Hemopoietic progenitors.* Filters contained on average 21% LTC-IC and 15% CFU-F of the total progenitor cell content. *Patients outcome.* Filter-discarded transplants had significantly more grade II-IV GvHD (42% vs 15%, p= 0,008) as compared to filter-recovered transplants, and more TRM (20% vs 3%, p=0.04). The actuarial survival at 5 years is 69% vs 87% respectively (p=0,15). *Conclusions.* This study suggests that (1) a significant proportion of LTC-IC are lost in the filters together with CFU-F; (2) recovery and add back of progenitors trapped in the filters may reduce GVHD and transplant related mortality.

0305

EXTRACORPOREAL PHOTOPHERESIS FOR TREATMENT OF FASCIITIS IN CHRONIC GRAFT-VERSUS-HOST DISEASE: A PILOT STUDY

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Background. Fasciitis is a rare, late onset manifestation of chronic Graft-versus-Host Disease (cGvHD) after allogeneic hematopoietic cell transplantation. It is characterized by condensed and hardened subcutaneous and fascial tissues, as opposed to cutaneous sclerosis. On biopsy, edema and fibrosis of the fasciae and intermediate septa with pericapillary lymphocytic infiltrates are found, without involvement of the muscle itself. Fasciitis of cGvHD affects predominantly the limbs, but involvement of the trunk leading to respiratory compromise may also occur. Possible associated laboratory features include eosinophilia and elevated antinuclear antibodies (ANA). The clinical diagnosis can be confirmed by biopsy and / or magnetic resonance imaging (MRI). Physical therapy to prevent joint contractures is the mainstay of conservative treatment; typically, conventional immunosuppressive therapies fail or yield incomplete responses. Extracorporeal photopheresis (or photochemotherapy, ECP) is an attractive alternative for patients with fasciitis of cGvHD because of its systemic action and steroid-sparing effects. Although still not fully understood, tolerance induction through *ex vivo* psoralen-sensitized and UVA-irradiated T-cells is now believed to be the central mechanism of action of ECP. PATIENTS AND *Methods.* Here we report our seven years experience (2/1999-2/2006) of ECP in 16 consecutive patients with fasciitis of cGvHD: 4 females and 12 males; age 24-63 (median: 44) years; 5 AML, 4 ALL, 3 CML, 1 MDS and 3 lymphoma patients; 13 with matched related and 3 with matched unrelated donors; 4 patients with reduced intensity and 12 with myeloablative conditioning. All patients had severe, extensive cGvHD and MRI- or biopsy-proven fasciitis. Diagnosis was made 3-28 (median: 15) months after transplantation or donor lymphocyte infusions (DLI), respectively. ECP was performed with the UVAR XTS machine (Therakos, Exton, PA, USA): one cycle consisting of two consecutive sessions every two to four weeks according to the clinical course. *Results.* After 4-82 (median: 17) months of ECP therapy, 13 of the 16 patients (81%) had marked (7 patients) or moderate (6 patients) clinical amelioration in their range of movement, whereas 3 patients experienced no change with ECP. Furthermore, all responders were able to considerably reduce (7 patients) or discontinue (6 patients) their immunosuppressive medication. Responses were first noticeable after five to six ECP cycles and continued to improve with maintenance therapy. When a patient's maximum achievable improvement had been reached, the frequency of ECP cycles could be tapered to once every four to six months, or ECP could be discontinued altogether. ECP was well tolerated without major toxicities or infectious complications. In comparison, among eight historical or contemporary control patients treated by standard immunosuppressive agents, who had not received ECP for reasons of patient preference or unsuitable venous access, only two recovered (one with steroids and one with PUVA therapy), whereas five had no change and one deteriorated. *Conclusion.* In our experience, ECP is a safe and effective immunomodulating approach for patients with fasciitis of cGvHD after allogeneic hematopoietic cell transplantation. Prospective evaluation of ECP in fasciitis of cGvHD in a multicenter trial would be warranted.

0306

REDUCED INTENSITY CONDITIONING (RIC) IN ALLO-BMT FOR RESISTANT-RELAPSED LYMPHOMAS

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Background. The use of RIC conditioning regimen is now largely applied in conditioning regimen for TMO in haematological malignancies however no definite indications exist on which patients may be benefited by this procedure although most of experiences are focused on patients with lymphomas resistant or relapsed to other therapies. *Aims.* To disclose if RIC may be a therapeutic option as salvage treatment with acceptable results in a population of relapsed-resistant patients with lymphomas, mostly already treated by high-dose therapy and PBSCT. The inclusion in the RIC procedure of those patients with age over 60 years leads to valuate if the toxicity, namely related to TRM, is acceptable. *Methods.* We present a cooperative experience on RIC focused on 42 advanced, relapsed and resistant lymphomas to several therapies including high dose and autologous PBSCT. The mean age of patients was 49 years (23-67 years). RIC consisted in the combination of TT 10 mg/Kg, Fludarabine 50 mg×5 and CTX 300 mg×5 for 27 patients (64%) and other similar combinations for the other patients. Diseases included 13 HD resistant, 9 HGBL, 5 FL, 6 HGTL, 4 CLL, 2 ALC and 3 LPL relapsed and

resistant to a series of therapies. Thirty-one patients have been already treated by high-dose therapy and PBSCT. *Results.* Thirty-six patients (86%) are valuable for response, 6 patients died within 3 months from RIC for causes not related to disease progression (TRM=14%). The response valuated within 6 months from RIC showed 26 pts in CR (72%) and 10 patients with persistence of disease (28%), 3 relapses (14%), were registered following 12, 18 and 24 months from RIC. Eight patients died (22%) for disease progression among valuable pts, one further death was registered for chronic GVHD 18 months following RIC accounting at 15 the total number of deaths including TRM (36%). Five patients are alive with lymphoma and 22 alive in CR (52%) at a mean follow-up of 28 months (7-59 months). The incidence of GVHD was registered in 16 patients (38%) and 6 of them had grade 3-4. This was not correlated to previous therapy or to type of RIC conditioning regimen or to the age of patients. Deaths accounted 11 on 31 (35%) patients who received autologous PBSCT and 4 on 11 (36%) on those who did not. *Conclusions.* RIC transplantation provides an high rate of remissions in patients with advanced lymphoma and an acceptable TRM. The results are independent from the previous therapy and type of disease; the incidence of GVHD and relapses are not correlated to different conditioning regimen. Future prospective trials including RIC transplantation are planned.

0307

FLUDARABINE BASED REDUCED INTENSITY CONDITIONING FOR ALLOGENEIC TRANSPLANTATION IN PATIENTS WITH NON-MALIGNANT HEMATOLOGICAL DISORDERS

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Patients who are multiply transfused or septic have a poor outcome after allogeneic stem cell transplantation. Seventy patients (53 males and 17 females) with non-malignant disorders underwent allogeneic BMT using a fludarabine based conditioning regimen between 1998 and 2005. The median age was 20 years (range: 4-38) and consisted of 25 children and 45 adults. Indications for BMT included severe aplastic anemia (SAA) in 54, Myelodysplastic syndromes (MDS) in 8, Fanconi's anemia (FA) in 6 and Thalassaemia in 2 patients. All had 6 antigen matched sibling or family donors. Multiple transfusions (>20), sepsis or previous immunosuppressive therapy were considered high risk (HR) and 51 patients (72.8%) were considered high risk patients. The median time from diagnosis to transplant was 16 months (range: 2-108) and the median transfusions prior to BMT was 35 (range: 2-380). Conditioning therapy included Fludarabine (Flu) 180 mg/m^2 over 6 days, Busulfan (Bu) 8 mg/kg over 2 days and ATG 40 mg/kg/day over 4 days(24), Flu 180 mg/m^2 over 6 days, Cyclophosphamide (Cy) 120 mg/kg over 2 days + ATG 40 mg/kg/day over 4 days(35), Flu 180 mg/m^2 over 6 days, Cy 20 mg/kg over 2 days + ATG 40 mg/kg/day over 4 days(6), Flu/TBI/OKT3 in 4, Flu/Mel in 1. Graft versus host disease (GVHD) prophylaxis consisted of Cyclosporine alone or in combination with mini methotrexate. Graft source was peripheral blood stem cells in 56 patients and G-CSF stimulated bone marrow in 14. The median cell dose was 5.4×10^8 MNC/kg (range: 2.1-13.6) for PBSC and 6.2×10^8 TNC/kg (range: 2.1-16) for bone marrow. Nine patients expired within the first 10 days due to sepsis. 59/61 (96.7%) patients engrafted with a mean time to ANC > 500 of 12.2 days (range: 8 - 29), and platelet count > 20,000 of 14.2 days (range: 0 - 32). Two patients had primary graft failure and expired. Acute GVHD was seen in 18 patients (30.5%) with Grade III-IV GVHD in 6 (10.1%). Chronic GVHD was seen in 14 patients (29.1%) with 9 having limited and 5 with extensive GVHD. Bacterial infections were seen in 16 patients, fungal infections in 19 and CMV in 8 patients. Veno-occlusive disease was seen in 5 patients (7.1%) while hemorrhagic cystitis was seen in 3 (4.2%). Four patients (2 with aplastic anemia and 2 with thalassaemia) had secondary graft rejection. Day 100 mortality was 28% and was related mainly to sepsis. At a median follow up of 20 months (range: 2-84); 46 patients (65.7%) are alive with 44 patients (62.8%) being free of disease. Among patients who were low risk, 17/19 (89.4%) are alive and free of disease. The disease free survival was 66.6% in SAA, 62.5% with MDS, 50% with FA and 0% with Thalassaemia. In conclusion fludarabine based conditioning regimen ensure adequate engraftment with reduced toxicity in high risk patients who are infected or multiply transfused at the time of BMT. Its role even in good risk patients needs to be further explored.

0308

SAFETY AND EFFICACY OF BORTEZOMIB IN RELAPSED MULTIPLE MYELOMA FOLLOWING REDUCED INTENSITY/NONMYELOABLATIVE CONDITIONINGS AND ALLOGENEIC HAEMATOPOIETIC CELL TRANSPLANTATION

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Background. Despite the promising results obtained in patients with multiple myeloma (MM) after reduced intensity/nonmyeloablative conditioning regimen and allogeneic haematopoietic cell transplantation (HCT), relapse remains an issue. Several clinical trials showed the efficacy of bortezomib in the treatment of refractory/relapsed MM, by inhibition of NF-kB. These findings and the demonstration of the role of NF-kB in the pathophysiology of graft-versus-host disease (GVHD) provide the rational for using bortezomib in patients with MM relapsed after allogeneic HCT. *Aims.* We evaluated safety and efficacy of bortezomib after reduced intensity/nonmyeloablative conditioning regimen and allografting. *Methods.* We retrospectively evaluated 24 myeloma patients relapsed after allografting. Conditioning regimens were 2 Gy total body irradiation based in 19 patients and, a combination of thiotepa, cyclofosmide, and melphalan in 5. Donors were HLA identical siblings in 22 patients, and unrelated in 2. All patients received cyclosporine as part of post-grafting immunosuppression. Bortezomib was administered after a median of 20 months from HCT (range 5-54) and 34 months from diagnosis (range 19-164): 6 patients were in first relapse after HCT, 10 patients in second and 8 beyond the third relaspe. Patients received bortezomib 1.0 (n=8) or 1.3 mg/m^2 (n=16) on day 1, 4, 8, 11 every 3 weeks for a median of 3 courses (1-7), alone (n=5) or in combination with dexamethasone 20 (n=13) or 40 mg (n=5) on days 1-4 and 15-18 or daily prednisone 75 mg (n=1). No patient was on cyclosporin or thalidomide, and none had active GVHD at the time of administration. *Results.* Adverse effects were reported in 75% (18/24) of patients: thrombocitopenia was observed in 33% (8/24; >grade 3 5/8), peripheral neuropathy in 58% (14/24; >grade 3 5/14). Three additional patients experienced grade 2 urticaria, grade 2 liver toxicity and grade 3 neutropenia, respectively. Bortezomib was discontinued after the first cycle in 4 patients due to neurological toxicity, and in 2 patients for disease progression. A dose reduction was required in 3 patients due to neurological toxicity. Flaring of prior chronic limited GVHD was observed in one patient who developed mild liver GVHD. After a median follow up of 136 days (range 42-502), 21/24 patients are alive. Two non-responsive patients and 1 responsive patient died from disease progression. Among patients who completed at least 2 courses, overall response was 67% (12/18) including 5 immunofixation-negative complete remissions. No significant differences in toxicity and response rates were seen between bortezomib plus steroids and bortezomib alone. *Conclusions.* Bortezomib is capable of inducing disease responses in patients with MM relapsed after allogeneic transplant. No significant effect on GVHD was noted. Interestingly, in this subset we observed a higher incidence of peripheral neuropathy compare to the non-transplant population, which may be related to previous prolonged treatment with high dose cyclosporine. Longer follow up will demonstrate whether remissions will be durable.

0309

CYTOKINES AND T-CELL SUBSETS CHANGE IN PATIENTS HAVING GRAFT-VERSUS-HOST DISEASE

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Background. GVHD is the consequence of the activation of donor T-lymphocytes attack the tissue of host. Animal studies strongly suggest that T-cell activation in patients with acute GVHD have a CD4 subset imbalance favoring T helper 1 (Th1), which secrete type 1 cytokines interleukin (IL)-2, IL-12, interferon (INF)-γ, and TNF-α. On the other hand, the polarization toward Th2, which secrete type 2 cytokines IL-4 and IL-10, and subsequent Th2 humoral immune response may be responsible for the development of chronic GVHD. Both Th1 and Th2 are derived from naïve T cells and most clearly defined differentiation inducers are themselves cytokines: INF-γ and IL-12 for Th1, and IL-4

and IL-10 for Th2. Understanding the cytokines and T cell subsets change in patients with GVHD will theoretically of great help in elucidating the pathophysiology of GVHD. *Aim.* To see the cytokines and T cell subset changes in patients having acute and/or chronic GVHD. *Methods.* Consecutive 23 patients received allogeneic hematopoietic stem cell transplantation at China Medical University Hospital were enrolled in this study. 10 mL peripheral blood was collected every 7 days from Day 7 after transplantation till Day 200 (or Day 300 for patients with chronic GVHD). Plasma level of INF-γ, IL-4; IL-10, and IL-12 were determined by ELISA (R&D, Minneapolis, MN, US). Flow cytometric analysis of intracellular INF-γ and IL-4 in mononuclear cells with or without phorbol 12-myristate 13-acetate [PMA] + ionomycin [I] stimulation was used to determine the relative fraction of Th1/Th2 subset. The serial plasma level of each cytokine and relative fraction of Th1/Th2 were then compared to the clinical events in each patient. *Results.* Plasma IL-10 level increased markedly during period of both acute and chronic GVHD. Plasma INF-γ level also increased in most events of acute and chronic GVHD. With effective immunosuppressive therapy, plasma IL-10 and INF-γ level decreased rapidly, Plasma IL-4 and IL-12 were below the detectable level (0.13 pg/mL and 0.5 pg/mL respectively) in most patients, even during period of severe GVHD. Figure 1 demonstrates the correlation between plasma level of each cytokine (INF-γ, IL-4; IL-10, and IL-12) and clinical course of a patient with both acute and chronic GVHD involving liver. Flow cytometric analysis showed that Th1 (CD4+INF-γ+) fraction increased markedly within the CD4+ T cell population during period of both acute and chronic GVHD. Of great interesting is that the CD4+INF-γ+ T cells can be easily detected in the blood of many patients of GVHD without adding *PMA+I* to stimulate T cells. *Conclusion.* Immune reactions in patients of GVHD are much more complicated than in animal models. Type 1 cytokine (INF-γ) and type 2 cytokine (IL-10) may be increased in the blood at the same time during acute and chronic GVHD. Increased Th1 fraction could also be found during both acute and chronic GVHD. Th1 as well as IL-10 and INF-γ may therefore play an important role in the pathogenesis of both acute and chronic GVHD. Besides, they may also be served as good biomarkers in monitoring the clinical course of GVHD.

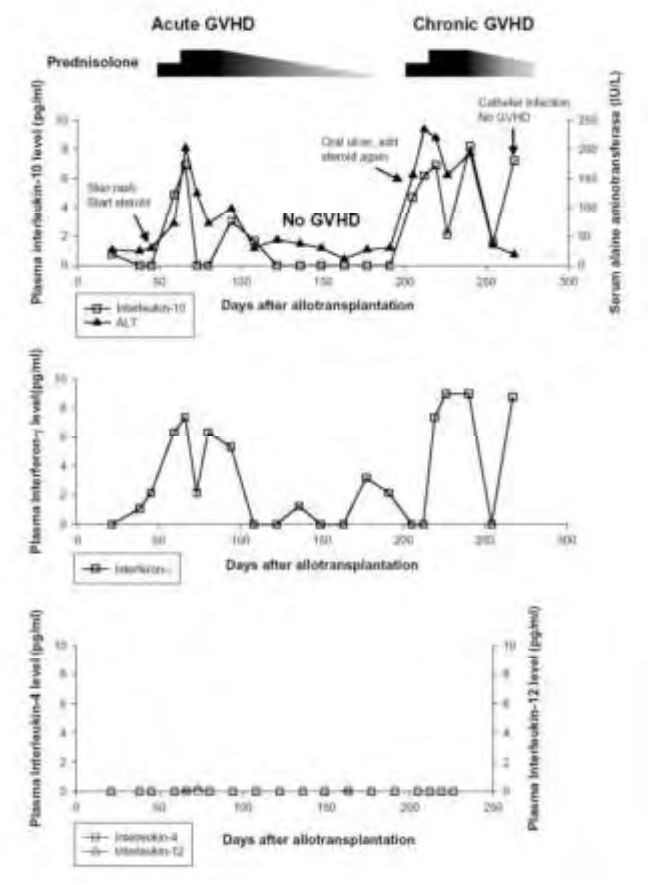

Figure 1. Cytokines' change and clinical course of GVHD

0310

NCREASED INCIDENCE OF CYTOMEGALOVIRUS RETINITIS AFTER ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Cytomegalovirus (CMV) has been recognized as a major cause of morbidity and mortality after allogeneic hematopoietic stem cell transplantation (HSCT). CMV retinitis (CMVR), frequent in patients with acquired immunodeficiency syndrome but rarely involved in HSCT recipients, can lead to retinal destruction and blindness if untreated. We observed 6 cases from 2002 to 2005 in our center whereas only one case was diagnosed from 1985 to 2001. We described clinical and biological features of patients who developed a CMVR from 2002 to 2005 and determined incidence and risk factors of CMVR. Among 312 patients, who received HSCT in our center from 2002 to 2005, 117 had CMV reactivation and 24 had at least one episode of CMV disease. Of the 24 patients with CMV disease, 6 had CMV retinitis. Cumulative incidences were determined with death as competing event and risk factors (SPLUS 2000 Software). The Cox proportional hazard regression model was used to test significance of covariate. Of the six patients with CMVR, five received an HLA-identical bone marrow transplant from a related (n=1) or an unrelated (n=4) donor. The other patient received an unrelated HLA mismatched cord blood (UCB). CMVR was diagnosed in median 152 days after HSCT either on visual symptoms (n=3) or on a systematic ophthalmologic examination (n=3). All patients experienced at least 2 CMV reactivations prior to retinitis diagnosis and one patient had a previous CMV disease. The median lymphocyte count at diagnosis was 0.5×10^9/L (range: 0.32 to 1.21×10^9/L), the CD4 count was lower than 0.2×10^9/L in all patients and lower than 0.05×10^9/L in all but one. Retinitis resolved with a systemic intravenous antiviral treatment (foscarnet or gancyclovir) in all treated patient. One patient remained with sequelar visual trouble. Three patients relapsed from retinitis and were successfully treated again by intravenous antiviral treatment. Three-year cumulative incidence of CMVR was 2.2% among all transplanted patients, 3.5% in CMV-seropositive recipients and 6.5% in CMV-seropositive recipients transplanted with a CMV-seronegative donor. The combination of a CMV-seropositive recipient and CMV-seronegative donor was the only risk factor found in our study. The source of stem cell, conditioning regimen, the use of antithymoglobulin, age and GVHD were not related to an increased risk of CMV retinitis but small number of CMVR limited power of the analysis. We observed an increased incidence of CMVR compared with incidence before 2002 in our center and published incidence < 0.5% (Crippa F. CID 2001). This increase could be explained by a change in HSCT recipient management: improvement in supportive care, antiCMV pre-emptive therapy and increase in proportion of unrelated donor as well as cord blood. Finally, in patients with multiple CMV reactivations, we suggest to practice regular ophthalmologic examination in order to diagnose retinitis before visual trouble.

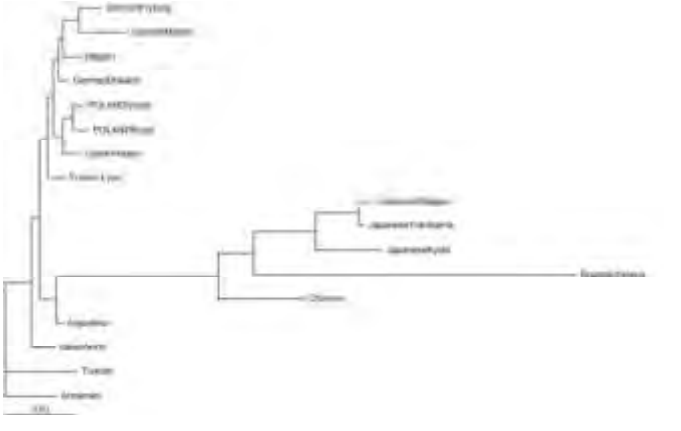

Figure 1. Neighbour-joining tree of Polish and other populs.

0311

STEM CELL DONORS RECRUITED FROM RURAL POPULATIONS HAVE A POTENTIAL TO INCREASE POLYMORPHISM OF UNRELATED DONOR REGISTRIES

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Background. Availability of matched stem cell donor is limiting factor for transplantations to patients who might benefit from this therapy. At most, 25% of patients may be supported by family donors and 30-50% of them receive stem cells from unrelated or other alternative donors. For the remaining patients no suitable donor is available because of unacceptable HLA mismatches. Several approaches are undertaken to increase HLA polymorphism of unrelated donor registries. Preferential typing of ethnical minorities or further typing of donors with rare phenotypes were used optionally. *Aims.* The aim of this study was to show the structure and genetic differences between urban and rural Polish subpopulations and to check the utility of dispersed rural population for increasing of unrelated donor registry HLA polymorphism. *Methods.* Five HLA loci (A, Cw, B, DRB1 and DQB1) were DNA typed at allele (4 digit) level in Polish population. The analysis comprised 200 unbiased, healthy individuals living in cities with >100 000 citizens (urban, N=106) and those living out of big cities (rural, N=94). The genetic structure measures of these two Polish subpopulations and five locus phylogenies along with other European, Mediterranean and Far-Eastern populations were analysed. *Results.* All loci were in Hardy-Weinberg equilibrium in both subpopulations (p>.05). A significant heterozygote excess was confirmed for DQB1 (p=.014, SE=0.001) and globally (p=0.028, SE=0.009) in rural sample and on the contrary, global heterozygote deficit was found in urban population (p=0.039, SE=0.012). As could be expected estimates of Nm, the number of migrants exchanged per generation, appeared to be high (Nm=19.60) after correction for sample size (mean sample size, N=100), suggesting high gene flow between two populations caused predominantly by country-to-city migration. Genic and genotypic differentiation tests revealed that none of five loci differentiated the two samples (p=.85 and p=.82 respectively), confirmed by low Fst values (<0.002). Although similarities between the two samples were obvious, some alleles were met exclusively in one of them. The polymorphism of urban subpopulation seemed to be slightly higher (121 and 114 HLA alleles in urban and rural population respectively) but we revealed A*2608, 2902, 3301, 6802, Cw*0804, B*0704, 1503, 4501, 4507, 5301, DRB1*0103 and DQB1*0304 only in rural sample. Some allele frequencies (AF) differed significantly between groups. AF of A*3201, B*3501, DRB1*0801 and DQB1*0302 were much higher in urban sample (3.7, 6.3, 5.2 and 8.1% respectively) than in rural sample (1.7, 3.0, 2.2 and 3.9% respectively) while those of the B*4402 and DQB1*0603 were much higher in rural sample (6.2 and 8.0% respectively) than in urban sample (3.6 and 3.9% respectively). This heterogeneity did not influence much the phylogenetic analysis, which showed the close relationship of both Polish subpopulations clustering along with Czech population to *Slavic branch*. Clear-cut departure of Polish subpopulations from *Far-Eastern branch* and associations with Western-European populations can also be concluded from the neighbour-joining tree. *Conclusions.* The present study revealed panmictic rather than differentiated structure of urban and rural Polish subpopulations. Nevertheless, rural population remains a reservoir of exclusive genotypes that potentially can increase registry polymorphism.

0312

POOR PROGNOSIS FOR PATIENTS AFTER MYELO- AND NON-MYELOABLATIVE CONDITIONING THERAPY FOLLOWED BY ALLOGENEIC PERIPHERAL BLOOD STEM CELL TRANSPLANTATION ADMITTED TO INTENSIVE CARE UNIT

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Background and Aims. The role of intensive care unit (ICU) support for patients following allogeneic peripheral blood stem cell transplantation (PBSCT) is controversial. In an era of constrained resources, we assessed prognostic factors predictive for survival in patients after myeloablative (MAC) and non-myeloablative allogeneic (non MAC) PBSCT over a period of 5 years. *Patients.* Between January 2000 and February 2005 two hundred and nineteen patients with various hematological malignancies underwent allogeneic stem cell tranplantation in our institution (MAC 142,non MAC 77). Of these, fourty-nine patients (22,4%) with a median age of 47 years (range 18-64 years; female 18, male 31; MAC 34, non MAC 15) were admitted to the ICU during the first two years following PBSCT (median 53 days, range 1-613 days). We looked for variables defining the SOFA (Sequential Organ Failure Assessment) and the SAPS (Simplified Acute Physiology Score) score on the day of ICU admission and six days later to discriminate patients with poor and good prognosis with regard to survival. We also looked for variables such as age of patients, diagnosis, disease status, donor type, time between transplantation and ICU admission, reason for ICU admission and occurrence of veno-occlusive disease and GVHD. *Results.* Mechanical ventilation was necessary in all patients admitted to the ICU. Median survival following referral to ICU was 29 days (range 5-959 days). The main reason for death was sepsis (51%). Nine of 34 patients (26%) who had received MAC survived the ICU stay with a median survival time of 11 months (range 2-29 months). In the group of patients who had received non-MAC three out of 15 patients (20%) could be discharged from the ICU with a median survival time of 5 months (range 4-12 months). Looking at the twelve patients in total there were three patients who survived the following year resulting in an overall survival of 6% one year after ICU admission. Only the SOFA score (p=0.002) on the day of ICU admission was of prognostic relevance for survival. *Conclusion.* ICU admission and respiratory failure are associated with poor prognosis after allogeneic stem cell transplantation. The probability of survival is independent from the type of conditioning therapy. The SOFA score is a predictor for short term survival but fails to identify long term survivors.

0313

HEMATOPOIETIC STEM CELL (HSC) RECRUITMENT HAS AN INFLUENCE ON TRANSPLANT OUTCOME AFTER REDUCED INTENSITY ALLOGENEIC PERIPHERAL BLOOD STEM CELL TRANSPLANTATION (PBSCT): A STUDY OF THE SOCIETE FRANCAISE DE GREFFE DE MOELLE OSSEUSE ET DE THERAPIE CELLULAIRE (SFGM-TC) REGISTRY

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Impact of graft product on transplant outcome after PBSCT is actually demonstrated. We investigated retrospectively the potential impact of HSC recruitment procedure (i.e. G-CSF stimulation schedule and apheresis number) and graft composition (CD34+ and CD3+ cell number) on transplant outcome (GVHD, OS, EFS). Our analysis concerned 488 HLA matched sibling allogeneic reduced intensity conditioning (RIC) PBSCT for haematological malignancies (116 MM, 110 AML, 109 NHL, 41 CLL, 41 MDS, 24 CML, 19 HD, 17 ALL and 11 MPS) reported on the SFGM-TC registry between 1998 and 2004. RIC-PBSCT was performed during first line treatment in 225 (49%) patients and a previous HSCT was recorded in 55% of the cases. Before RICT, 161 patients were in Complete Response, 161 in Partial Response, 34 in Stable Disease and 132 in Progressive Disease. Follow-up was updated in April 2005. G-CSF median duration was 5 days (3-7days) at a median dose of 10µg/kg/day (4.6-16). G-CSF was given bid in 40% of the stimulations. Filgrastim was used in 59% of the donors (Lenograstim: 41%). Only 107 donors (22%) had a single apheresis. The median number of CD34+ cells infused was 5.6×10^6 CD34+/kg (1-26) and the median CD3+ cells was 302×10^6 CD3+/kg (63-996). Conditioning regimen was most frequently an association of Fludarabine Busulfan and Anti Thymocyte Globuline (246 cases, duration of ATG 1 day: 18%, 2 days: 20%, 3 days: 20%, 4 days: 8% 5 days: 33%) or Fludarabine + TBI 2 Gy (123 patients). GVHD prophylaxis was a cyclosporine based treatment in 478 (95%) patients. Median follow-up after transplantation was 35 months (range: 0-86). Acute GVHD (grade II-IV) and cGVHD incidences were 35% (n=163) and 50% (n=217 for 430 patients) respectively. The 3-year OS was 40% and the 3-year EFS was 34%. Treatment related mortality was 15% at 3 years. In multivariate analysis studying pre and post transplant factors a significant impact was shown of G-CSF duration (HR: 0.79 (0.62-1) p=0.05), G-CSF daily dose (HR: 1.13 (1-1.28) p=0.04) on OS and a trend for G-CSF dose on EFS (HR: 1.1 (0.97-1.25) p=0.12). Other variables also influenced OS (NHL vs AML, aGVHD grade II vs 0-I and III-IV vs 0-I and cGVHD: yes vs no) and on EFS (Sex mismatch, ABO incompatibility, NHL vs AML, FBS ATG duration: 5 days vs 2 days, aGVHD grade II vs 0-I and III-IV vs 0-I and cGVHD: yes vs no). No influence of graft composition or stem cell recruitment was demonstrated on incidence and severity of aGVHD and cGVHD although we found a significant impact of conditioning (FBS ATG 1 day vs 2days and Fluda-TBI vs FBS ATG 2days). In conclusion, this study demonstrates that, surprisingly, graft composition has no impact on transplant outcome. Prolonged administration of moderate dose of G-CSF seems to be the best schedule for PBSC recruitment.

0314

TACROLIMUS AND MYCOPHENOLATE MOFETIL FOR GVHD PROPHYLAXIS AFTER ALLOGENEIC STEM CELL TRANSPLANTATION FOLLOWING REDUCED INTENSITY CONDITIONING

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Background and Aims. The combination of tacrolimus (TAC) and mycophenolatemofetil (MMF) is widely used to prevent rejection after solid organ transplantation. We here present our single center experience with this combination therapy after allogeneic blood stem cell transplantation. *Methods.* Forty patients (24 male, 18 female, median age 52 years, range 26-66) with advanced hematologic malignancies (10 AML, 2 ALL, 4 CML, 4 MDS, 4 MM, 15 Lymphoma, 1 RCC) and 2 with vsAA were given blood stem cells (bone marrow 5, PBSCs 37, median 8×10^6 34+ cells/kg, range 1.4-25) from related (4) or unrelated (38) donors after conditioning with fludarabine (3×30 mg/m^2) and low dose TBI (2Gy). Two patients with aplastic anemia received 100mg/kg cyclophosphamide in addition. All patients were treated with tacrolimus and MMF (TAC/MMF) to facilitate engraftment and prevent GvHD. Ten patients (24%) received campath 1H in addition and were scheduled for preemptive donor lymphocyte infusions. All patients had high risk disease as defined by the failure of previous therapies or unfavourable karyotype, were heavily pretreated and had contraindications against a myeloablative conditioning regimen. Twenty-one patients (50%) had active disease at transplantation. *Results.* Thirty-seven patients (88%) had stable donor cell engraftment, while 3 patients had primary and 2 had secondary rejection after initial donor cell engraftment. Both patients with vsAA engrafted with marrow. Sixty-seven percent of patients with active disease had objective responses. Acute GvHD occurred in 55% (16× grade I-II, 6× grade III-IV) and 16 of 34 evaluable patients (47%) developed chronic GvHD (9× limited, 7× extensive). Sixteen patients (38%) had disease progression or relapsed and 12 of them (29%) died of their original disease. On the other hand 12 patients (29%) died after a median of 257 days (range 31-616) because of treatment related causes, which were infection with (7) or without (5) GvHD. After a median follow up of 747 days (range 132-1800) 17 patients are alive (43%) in remission (43%, 15 CR, 2 PR) and 1 (RCC) with progressive disease. Major side effects of TAC/MMF were immunosuppression, infection, renal failure and GI disturbances. One patient developed irreversible blindness on one eye during TAC/MMF and on the other after switching from TAC to CSA. *Summary and Conclusions.* In this very high risk patient group TAC/MMF was effective in promoting engraftment after minimal conditioning. GvHD was acceptable and similar to interantional data on CSA/MMF or CSA/MTX in related donors after reduced intensity conditioning suggesting that TAC/MMF has at least equivalent immunosuppressive activity. Still GvHD remains the major problem after reduced intensity conditioning urging the need for additional solutions.

0315

COMPARATIVE OUTCOMES OF FLUDARABINE-BASED NONABLATIVE AND ABLATIVE CONDITIONING FOR PATIENTS WITH ADVANCED HEMATOLOGIC MALIGNANCIES

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Background. The role of nonablative allogeneic transplantation is not defined for advanced hematologic malignancies. *Aims.* We have conducted a comparison of the outcomes of nonablative and ablative conditioning directly for the treatment of patients suffering from advanced hematological malignancies. *Methods.* Adult patients with advanced hematologic malignancies (n=75; acute leukemia beyond 1st remission, 56; chronic myeloid leukemia beyond 1st chronic phase, 6; refractory Non-Hodgkin's lymphoma, 10; refractory multiple myeloma, 3) received transplants from human leukocyte antigen-matched donors, either related or unrelated, coupled with either nonablative (n=40; fludarabine/melphalan, 28; fludarabine/cyclophosphamide, 12) or ablative conditioning (n=35, busufan/cyclophosphamide). The patients receiving nonablative conditioning were elderly, or exhibited contraindications for ablative conditioning. *Results.* Neutrophil engraftment (i.e., time to ANC>0.5×10^9/L) occurred more rapidly in the nonablative group (median, 9 days; range, 0-19 days) than in the ablative group (median, 18 days; range, 11-38 days)(p<0.0001). The time required to achieve a platelet count in excess of 20×10^9/L was 12 days (median; range, 7-28 days) in the nonablative group, and 22 days (median; range, 9-64 days) in the ablative group (p=0.0001). Acute graft-versus-host disease (>grade II) occurred at comparable frequencies in the nonablative and ablative groups (25% and 26%). Hepatic veno-occlusive disease developed in 1 patient (3%) in the nonablative group, and 7 patients (20%) in the ablative group (p=0.02). Day-100 and 1-year NRMs were 33% and 47% in the nonablative group patients, as compared with 38% and 56% in the ablative group patients (p=0.68). The overall 1-year survival rates of the nonablative and ablative group patients were 44% and 15%, respectively (p=0.16). *Conclusions.* We noted a clear trend toward a more favorable overall survival rate in the nonablative group patients. The results of this study indicate that patients suffering from advanced hematological malignancies might benefit from treatment via nonablative transplantation.

0316

IMMUNO HAEMATOLOGICAL RECONSTITUTION AFTER T-CELL-DEPLETED HLA-HAPLOIDENTICAL STEM CELL TRANSPLANTATION FOR THALASSEMIA

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Background. We evaluated haematological and immunological characteristics of four thalassemia patients after T-cell-depleted HLA-haploidentical stem cell transplantation. *Methods.* We evaluated the clonogenic capability by the colony forming cell assay (CFC) and the long term culture-initiating cell (LTC-IC) assay at baseline and 20 days after transplant. Stromal cells were obtained from long term culture of bone marrow mononuclear cells (BMMCs) and analysed by immunohystochemistry. Lymphocyte subsets were studied by flow cytometry; and stromal IL-7 production by BMMCs was analysed by ELISA. *Results.* At baseline, no significant differences were observed in haematological and in immunological parameters in thalassemia patients when compared with a group of normal subjects. Day + 20 after transplant, a reduced clonogenic capability was observed (4±2 vs. 41±40 CFU-E, 17±9 vs. 109±22 BFU-E, 3±1 vs. 9±6 CFU-GEMM and 16±10 vs. 66±23 CFU-GM). The number of primitive bone marrow (BM) progenitor cells was also decreased (1.8±1.4 vs. 15.4±3.6 LTC-CFC/106 BMMCs). In addition, stromal cells secreted lower IL-7 levels (0.3 + 0.1 pg/mL vs. 0.8 + 0.1 pg/mL, in controls) and displayed by immunohistochemistry an altered phenotype. Upon light microscopy examination, the majority (75%) of these cells appeared as moderately large cells, frequently rounded, with abundant cytoplasm, whereas in control subjects about 90% of the stromal cells exhibited a different morphology characterized by irregular or spindle shape and branching cytoplasmic processes (fibroblast-like). Compared with normal subjects, thalassemia patients showed: reduction of na_ve CD4+ T-cells (2±0.5% vs 50±10%), reduction of thymic na_ve CD4+ T-cells (1±0.2% vs 40±12%,) and a significant increase of CD4+ cells activation markers (CD95, HLA-DR and CCR5). IL-7 receptor (CD127) expression was also significantly decreased on CD4+ T-cells and on naïve CD4+ T-cells (CD4+/CD45RA+CD62L+/CD127+). NK cells were among the first lymphocytes to repopulate the peripheral blood, and up to 70% of these cells were CD56 brigh whereas CD16+ NK cells were decreased. *Conclusions.* Twenty days post transplant, an impaired growth and differentiation capacity of stem/progenitor cells were observed in thalassemia patients, in parallel with an altered homeostasis of T-cells and a reduction of T-cell naïve compartment. We hypothesize that the damage of T cell compartment may be at least partially due to an altered production of new T cells starting from the haematopoietic stem/progenitor cells. CD56+ NK cells develop more rapidly than other lymphocytes, but CD16+ NK cells (with cytotoxic potential) require more prolonged exposure to maturation factors (IL-2) in the bone marrow. An IL7/IL7R pathway dysregulation has been also observed, possibly involving bone marrow stromal cells. *in vitro* studies are ongoing about the use of cytokines (IL-2, IL-7, IL-2 plus IL-7) supporting T cell development.

0317

HIGH EFFICACY OF PULSE CYCLOPHOSPHAMIDE IN CORTICOSTEROID-REFRACTORY LIVER GRAFT-VERSUS-HOST DISEASE

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Background. Corticosteroid-refractory GvHD is difficult to manage, and is associated with high morbidity and mortality. Cyclophosphamide (Cy) is an established immunosuppressive and cytotoxic drug widely used as a part of conditioning regimens. Pulse Cy in the GvHD treatment is based on the Cy efficiency for the treatment of many autoimmune disorders and the autoimmune nature of GvHD. In our previous work, we showed that intestinal GvHD responded poorly to pulse Cy, whilst liver, skin and oral GvHD responded well. The liver GvHD is more frequent than other GvHD forms. *Aims.* We used pulse Cy in the treatment of corticosteroid-refractory liver GvHD with aims to evaluate efficacy, toxicity and influence of Cy to some clinically significant parameters. We analyzed our new data concerning liver GvHD. *Methods.* This is a retrospective study of 20 patients (pts) with hematological malignancies after allogeneic peripheral blood stem cell transplantation: 12 pts had acute GvHD (2 pts grade I, 3 pts grade II, 7 pts grade III), 4 pts had chronic extensive GvHD and 4 pts developed liver GvhD upon DLI. Three pts had only liver GvHD, 17 pts had GvHD with involvement of liver and/or oral mucosa, skin, gut. Nine patients had hepatitic variant of liver GvHD (serum aminotransferase ALT or AST elevation above 10 times the upper normal limit). All patients were treated by cyclosporine A and steroids in dose 2 mg/kg before pulse Cy, six patients had another previous therapy (mycophenolate mofetil, tacrolimus, ATG, alemtuzumab). Steroid-refractory GvhD was defined as lack or response to steroids administered for at least 5 consecutive days. Twenty pts with corticosteroid-refractory liver GvHD were treated by Cy at median dose of 1g/m^2 (range 460 mg/m^2-1500 mg/m^2). Sixteen patients received one pulse Cy, 4 patients two pulses of Cy. *Results.* There were 55% CR (11/20), 10% PR (2/20) and 35% NR (7/20). However, in 3 pts with NR their clinical status stabilized and they responded to another treatment. Eigth pts (89%) from nine pts with hepatitic variant of liver GvHD reached CR. Five pts died, 3 from intractable liver and intestinal GvHD, 1 from intestinal GvHD with liver GvHD in PR, and 1 from relaps of leukemia. No influence of pulse Cy to chimerism and disease status was observed. Leukopenia and/or thrombocytopenia WHO grade 4 developed in 5 patients. When myelosupression appeared, it was usually short-lived (1-4 days). Twelve infectious complications occurred in 8 of 20 pts (pneumonia 2×, febrile neutropenia 1×, CMV positivity 6×, BKV positivity 3x), all of them resolved after antimicrobial therapy. No other significance toxicity after Cy pulse was observed. Overall survival is 75%, with median and maximum follow-up of 12 and 58 months, respectively. *Conclusions.* Pulse Cy has a good toxicity profile and the cost of the drug is negligible. According to our results, pulse Cy is very effective therapy of steroid-refractory liver GvHD.

Apoptosis / Transcriptional control / Signalling

0318

HALOFUGINONE, INHIBITOR OF TRANSFORMING GROWTH FACTOR (TGF)B, INDUCES APOPTOSIS AND CELL CYCLE ARREST OF MULTIPLE MYELOMA CELLS *IN VITRO* AND IMPROVES HIND LIMB PARALYSIS IN THE 5T2 MM MOUSE MODEL *IN VIVO*

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Multiple myeloma (MM) is a devastating malignancy which remains incurable despite recent new novel therapeutic compounds. It was previously shown that Activin A, a member of the transforming growth factor (TGF)b superfamily, is a potent inhibitor of myeloma cells that act by blocking the cell cycle and inducing apoptosis. Halofuginone is a new novel inhibitor of TGFb signaling that works by inhibiting Smad3 phosphorylation. The purpose of this study was therefore to assess the effect of Halofuginone on MM cell lines *in vitro* and to evaluate its putative therapeutic potential using the mouse 5T2 MM tumor model that mimics human MM including bone lesions. The sensitivity of the MM cell lines to Halofuginone was monitored by the WST-1 viability assay, as well as by DNA fragmentation analysis, Annexin staining and cell cycle analysis. Halofuginone suppressed proliferation and induced apoptotic cell death of the MM cell lines in a dose dependent manner (IC50 varied between 15-200 nM). Incubation with Halofuginone resulted in cell shrinkage, chromatin condensation, nuclear and DNA fragmentation and Annexin staining. Cell cycle analysis showed induction of cell cycle arrest and cell death in Halofuginone treated MM cells. Finally, Halofuginone administration to the 5T2 MM mouse model resulted in reduction in hind limb paralysis and extended survival. In summary, Halofuginone induced cell cycle arrest and apoptotic cell death of MM cells *in vitro* and demonstrated an anti MM effect in the 5T2 mouse model *in vivo*. Therefore, Halofuginone may indeed have a therapeutic potential for MM.

0319

DOWNREGULATION OF RXRA EXPRESSION IS ESSENTIAL FOR THE DIFFERENTIATION AND PROLIFERATION OF NEUTROPHIL GRANULOCYTES

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Neutrophil granulocytes are short-lived leukocytes that have to be constantly regenerated from myeloid progenitors. Retinoid-X-receptor-α (RXRα) is the predominant RXR protein in myeloid cells. RXRα is able to heterodimerize with other nuclear receptor (NR) family members or form signal-competent homodimers. RXRα partner availability regulated by intracellular RXRα abundance is thought to determine NR signaling. However its regulation in primary neutrophil versus monocyte differentiation remained uncharacterized. Here we show that myeloid progenitors express RXRα protein at sustained high levels during M-CSF-induced monopoiesis. In sharp contrast, RXRα is downregulated during G-CSF induced late-stage neutrophil differentiation. Ectopic RXRα inhibited G-CSF-dependent cell proliferation of granulocyte progenitors as well as their differentiation to late stage LF+ neutrophils in a serum-free culture model of CD34+ human progenitors. Furthermore, ectopic RXRα was sufficient to redirect G-CSF stimulated progenitors to monocytes. In line with its elevation in monocytes, RXRα failed to inhibit, but rather augmented M-CSF-dependent monocyte generation. Functional genetic interference with RXRα signaling in hematopoietic progenitor/stem cells using a *dominant-negative* RXRα promoted the generation of late stage granulocytes *in vivo* and *in vitro*. Therefore, downregulation of RXRα protein is required for neutrophil generation. This differential regulation of RXRα protein expression is determined by granulocyte versus monocyte cytokine signals.

0320

TRAIL-R3 EXPRESSION ON MYELOID LEUKEMIC BLASTS IS RELATED TO SHORTENED OVERALL SURVIVAL

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Background. Since chemotherapy and transplantation can cure only around 35% of patients with acute myeloid leukemia (AML), there is still need for complementary and targeted treatment modalities. One of them could be the use of TNF-related apoptosis-inducing ligand (TRAIL). TRAIL is expressed by effector T cells and induces apoptosis via the death receptor intrinsic pathway. Activation of this pathway in addition to the mitochondrial pathway (by chemotherapy) has synergistic effects *in vitro*. In human, 4 membrane bound receptors have been identified: two of them (TRAIL-R1 (R1) and TRAIL-R2 (R2)) contain a functional death domain and are capable of starting the apoptotic cascade, and two others (TRAIL-R3 (R3)and TRAIL-R4 (R4)) lack a functional death domain and function as decoy receptors. Most normal cells express R3 and R4, where many tumor cells express R1 and R2. This makes soluble recombinant TRAIL an attractive candidate for targeted therapy; phase 1 clinical studies for solid tumors are launched. Until now, sparse data on TRAIL sensitivity of myeloid leukemic cells have demonstrated low TRAIL sensitivity. *Aims.* Investigation of possible role for TRAIL treatment in AML patients. *Methods.* We investigated blood and bone-marrow samples of 113 patients with AML for TRAIL receptor expression by flow-cytometry. Results were correlated to clinical data. Four myeloid leukemic cell lines with different expression levels of TRAIL receptors were tested for TRAIL sensitivity by treatment with soluble TRAIL. Downregulation of R3 expression was performed by treatment PI-PLC and cyclohexamide. *Results.* In contrast with published data, we found presumably (pro-apoptotic) R1 and R2 expression (mean percentage positive cells 16% and 34%, range 0-79% and 0-97% respectively) versus R3 and R4 expression (mean 9% and 10%, range 0-71% an 0-45%) indicating a TRAIL sensitive profile for myeloid blasts. Surprisingly, the expression of the anti-apoptotic R3 strongly correlated to survival. Expression of > 25% blasts positive for R3 resulted in shortened overall survival (p=0.0051), see figure 1. In multivariate analysis R3 expression remained a significant prognostic factor next to cytogenetics (p=0.03 and p=0.015 respectively). *In vitro* studies on myeloid leukemic cell lines confirmed TRAIL sensitivity in cell lines that expressed R1 and R2. Furthermore, simultaneous expression of R3 clearly reduced the amount of apoptosis, suggesting that TRAIL effects are inhibited by binding to R3. Removal of R3 by treatment with PI-PLC resulted in 50% reduction of R3 expression and partially restored TRAIL sensitivity *in vitro*. *Conclusions.* Our data suggest that, in contrast to earlier reports, there might be a role for TRAIL in apoptosis induction of AML blasts. R3 expression is a strong predictor for overall survival. In AML cell lines R3 expression resulted in less TRAIL sensitivity and removal of R3 partially restored TRAIL sensitivity. Modulation of R3 might yield additional new therapeutic options for AML patients.

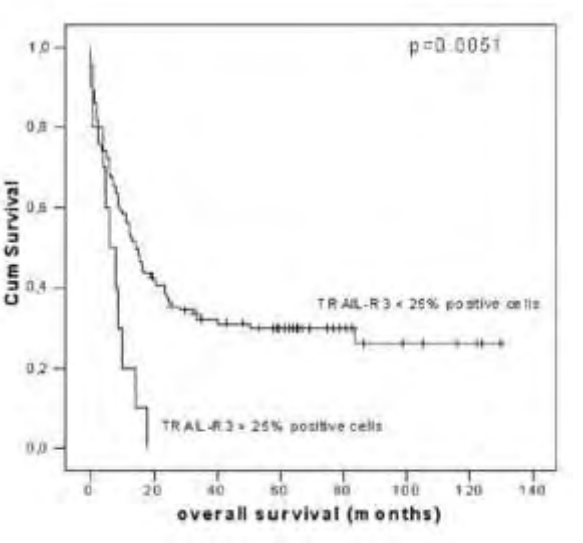

Figure 1.

0321

HTLV-1 PROPELS UNTRANSFORMED CD4+ LYMPHOCYTES INTO THE CELL CYCLE WHILE PROTECTING CD8+ CELLS FROM DEATH, AND ESTABLISHES A CD4+ RESTRICTED PRELEUKEMIC PHENOTYPE

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Background. HTLV-1 is the etiologic agent of adult T-cell leukemia/lymphoma (ATLL). *In vivo*, HTLV-1 infects both CD4+ and CD8+ lymphocytes, yet induces ATLL that is regularly of the CD4+ phenotype. *Aims.* To compare infection of CD4+ and CD8+ T cells by HTLV-1 *in vivo* and *ex vivo* in carriers without malignancy, and its implication in genesis of ATLL. *Methods. In vivo:* comparative analysis of proviral loads (real-time quantitative PCR) and clonality pattern (Inverse PCR) of highly purified CD4+ and CD8+ infected cells from 10 patients without malignancy. *ex vivo* : comparative analysis of 66 clones (infected versus uninfected / CD4+ versus CD8+) generated by limiting dilution from 4 infected patients. Monoclonality was confirmed by analysis of TCR-γ chain gene rearrangements of each clone (multiplex PCR-γ denaturating gradient gel electrophoresis analysis). Studied parameters : cell proliferation (cell count and 3H-thymidine incorporation, with and without interleukine-2), cell cycle (measurement of DNA content by flow cytometry after propidium iodide [PI] staining), apoptosis (flow cytometry after annexin V and PI staining), viral expression (ELISA and real-time quantitative RT-PCR) and cytology.

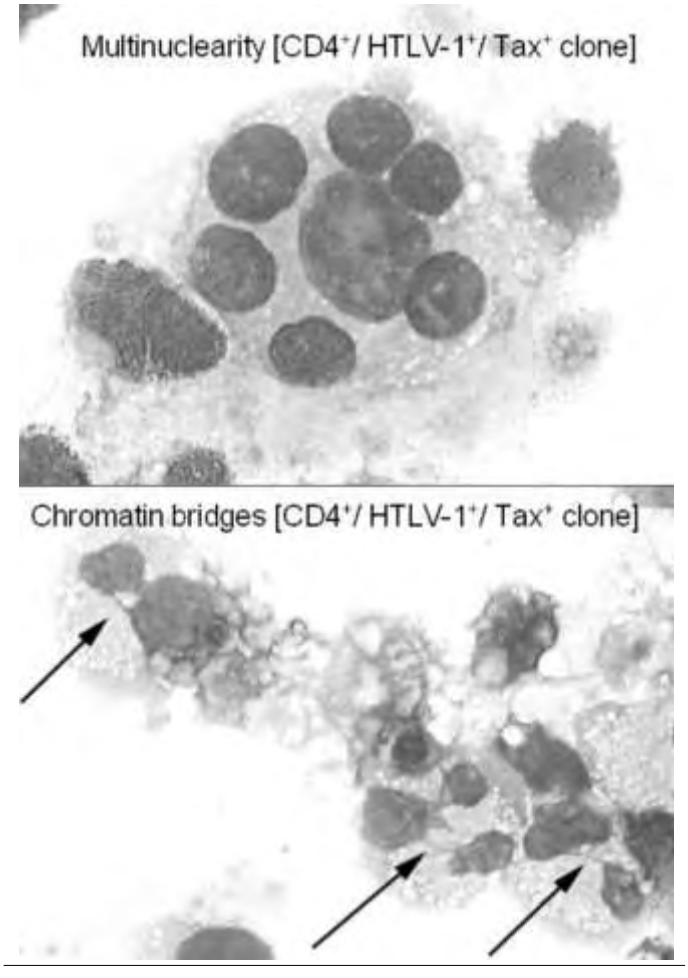

Figure 1. CD4+ restricted preleukemic cellular abnormalities

Results. Here we show that, *in vivo*, infected CD4+ and CD8+ T cells display similar patterns of clonal expansion in carriers without malignancy. Cloned infected cells from individuals without malignancy had a dramatic increase in spontaneous (without interleukine-2) proliferation, which predominated with CD8+ lymphocytes and depended on the amount of viral-encoded tax mRNA. In fact, the clonal expansion of HTLV-1 positive CD8+ and CD4+ lymphocytes relied on two distinct mechanisms: proliferation of CD4+ and accumulation of CD8+. This proliferation depended on the level of tax expression. Moreover, infected tax-expressing CD4+ lymphocytes cumulated cellular defects characteristic of genetic instability (dedifferentiation, multinuclearity and chromatin bridges) [image]. *Summary/Conclusions.* HTLV-1 infection establishes a preleukemic phenotype that is restricted to CD4+ infected clones. Finally, our results support that targeting CD4+ cell cycling is of interest in the prevention or treatment of ATLL.

0322

OLIGONUCLEOTIDE TARGETING OF THE BCL11B LOCUS CONTROL REGION REVEALS DISTINCT LEUKEMOGENIC ZONES AT DNASE-I HYPERSENSITIVE SITES

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Background. BCL11B is a translocation target in T-cell acute lymphoblastic leukemia (T-ALL) where it ectopically activates TLX3 or NKX2-5 homeobox genes via t(5;14)(q35;q32). Translocation breakpoints are distributed over the 1.2 Mbp *genomic desert* region downstream of BCL11B. *Aims.* To characterize this breakpoint region in order to identify regions and factors responsible for ectopic homeobox gene activation. *Methods.* We used cytogenetic, molecular and bioinformatic *Methods.* Fluorescence *in situ* hybridization (FISH), fiber-FISH and Halo-FISH; RT- and RQ-PCR, DNA-transfer by electroporation and lentivirus, gene expression knockdown by antisense oligos or siRNA, chromatin immunoprecipitation (ChIP); TRANSFAC database. *Results.* Using the TRANSFAC database we designed 26-mer double-stranded oligos (DSO) to constrain DNase-I hypersensitive sites (DHS) and other putative regulatory sites within the downstream BCL11B non-coding region by transfection of a T-ALL cell line (PEER) in which NKX2-5 is activated by insertion downstream of BCL11B. NKX2-5 modulation was regionally dependent, with 8/9 inhibitory DSO lying telomeric of the 14q32 breakpoint corresponding to regions displaying tight nuclear matrix attachment. DSO neighboring BCL11B itself inhibited that gene only, while the 4 DSO most efficacious against NKX2-5 lay adjacent to its insertion point at 14q32, matching both orphan DHS clusters and a desert acetylation island. ChIP analysis showed neither NKX2-5 nor TLX3 to be promoter-acetylated in T-ALL cells, unlike regions at efficacious inhibitory DSO. Expression of NKX2-5 and TLX3 in T-ALL cells was, nevertheless, sensitive to histone deacetylation inhibition implying their extrinsic regulation by acetylated factors. Gene knockdown studies identified PU.1 which is known to be regulated by acetylation as a key factor activating ectopic homeobox gene expression in T-ALL cells. TRANSFAC analysis revealed HMGA1 binding sites predominantly located near inhibitory DSO sequences. We discuss these results in the context of published interaction between PU.1 and HMGA1 and involvement of HMGA1 in enhanceosome structure/function. *Summary/Conclusion.* We have identified a distal leukemogenic regulatory hotspot downstream of BCL11B to serve as a potential therapeutic target within 'junk' DNA.

0323

ABSENCE OF SPRED1, A NEGATIVE REGULATOR OF TYROSINE KINASE ACTIVITY, IN CHRONIC MYELOID LEUKAEMIA PATIENTS

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Background. Spred1 proteins are inducible inhibitors of signalling induced by receptor tyrosine kinases. They are implicated in negative feedback interactions that regulate intracellular pathways. The repressive function of Spred proteins targets several TK receptors so resulting in a variety of biological effects. Spred proteins, after growth factor stimulation, translocate to the plasma membrane, become tyrosine phosphorilated and interact with components of the Ras/MAPK and Ras/Raf/Erk pathways. *Aim.* The aim of this study was to assess whether the activation of Bcr-Abl pathway leading to the disruption of many biological processes, could be supported by a defective signalling inhibition. *Methods.* Using a Real Time PCR we studied the expression lev-

el of Spred1 in 80 samples collected from CML patients at diagnosis (15 PB and 65 BM), and 9 BM samples from patients in blastic phase (BC). Furthermore, 12 CP patients were evaluated also at the time of the achievement of complete cytogenetic remission. Finally, 36 normal controls (20 PB and 16 BM) were studied. The protein level was analyzed by western blot and immunofluorescence assay. Sequence analysis of the coding and promoter regions was performed. In order to establish the effects induced of the absence of Spred1 on proliferation, we transfected K562 cells with Spred1 plasmid. After transfection colony growth was evaluated in semisolid medium, the proliferation rate was estimated by MTT assay and by the incorporation of 3H timidine. *Results.* We found that Spred1 transcript amount is significant reduced in CP CML samples (mean value of 2-ΔΔCt =0,02; range 0,1-0,0002) when compare to normal controls (mean 2,4) with a p value of 0,000002. This difference is even more sound in BC CML cells where Spred1 transcript is 4 logs lower compared to normal controls (2-ΔΔCt =0,0003 p=0,0000001). The expression levels significantly increased after reaching the cytogenetic remission (mean value of 2-ΔΔCt = 0,9; p=0,0007 compared to diagnosis) reaching values similar to normal controls (p=0,09). Western blot demonstrated the reduction or the absence of Spred1 protein in CML cells in CP and BC. By contrast, the protein reappeared after the achievement of cytogenetic remission. Sequence analysis allowed to exclude the presence of mutations in Spred1 coding and promoter regions. In order to better understand the mechanism leading to the abrogation of Spred1 we analyzed the factors responsible for Spred1 transcription. We demonstrated that the transcription factor WT1 binds to and activates the promoter region of Spred1. Moreover, we demonstrated a defective transcription activity of WT1 in CML patients due to the absence of one of the isoforms responsible for transcription. K562 cells transfected with Spred1 (K562+) showed a 55% reduction of the proliferation rate compared to untransfected K562 cells (K562-) Moreover a significant reduction of colony growth was observed in K562+ when compared to K562- (mean value of 25±7 vs 180±12). *Conclusions.* This study clearly demonstrates that the absence of Spred1 protein, a physiological inhibitor of RTK mediated signalling, is a common finding in CML cells and this may support the abnormal proliferation in Bcr-Abl positive cells.

0324

ROLE OF ID AND HES PROTEINS IN ACUTE PROMYELOCYTIC LEUKEMIA

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Acute promyelocytic leukemia (APL) is uniquely sensitive to treatment with all-trans retinoic acid (ATRA), which overcomes the differentiation arrest and induces terminal granulocytic differentiation of the leukemic blasts. In 98% of the cases of APL, the leukemic cells express a promyelocytic leukemia (PML)- retinoic acid receptor a (RARα) fusion protein as a result of a t(15;17) chromosome translocation. Previously, we have identified ID1 and ID2 as direct retinoic acid target genes. These proteins act as antagonists of basic helix-loop-helix (bHLH) transcription factors. ATRA induced a rapid, transient increase in ID1 and a sustained upregulation of ID2 both in the APL cell line NB4 as well as in primary leukemic cells from APL patients. To assess the relevance of this upregulation, ID1 and ID2 were overexpressed in NB4 cells. Overexpression inhibited proliferation and induced a G0/G1 accumulation. These results indicate that ID1 and ID2 are important retinoic acid responsive genes in APL. In addition, we studied another group of antagonists of bHLH transcription factors, the Hairy and Enhancer of split (HES) genes. We identified HES1, which is involved in Notch signalling, and has a very similar biochemical function as the ID-proteins, as a direct ATRA-responsive gene. In NB4 cells and in APL patient cells, ATRA induced a rapid but transient increase in HES1 followed by a sustained downregulation of HES1 expression. In the 5' upstream promoter we identified a retinoic-acid response element. Chromatin-immunoprecipitation assays revealed an interaction of PML-RARa with the HES1 promoter, suggesting a role for HES1 during ATRA-induced differentiation of APL cells. Overexpression of HES1 in APL cells will provide insight into the function of HES1 during APL cell proliferation and differentiation, and apoptosis.

0325

REGULATION OF AUTOPHAGIC PROGRAMMED CELL DEATH BY THE BALANCE BETWEEN CERAMIDE AND SPHINGOSINE-1-PHOSPHATE THROUGH MAMMALIAN TARGET OF RAPAMYCIN (MTOR) IN HUMAN LEUKEMIA HL-60 CELLS

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Background and Aim. The balance between ceramide and sphingosine-1-phosphate has been suggested to be critical to cell death and survival in the fate of leukemia cells. Autophagy is recognized as one of the important mechanisms in the metabolization of cellular components, and has recently emerged as a caspase-independent programmed cell death (PCD) system different from classic apoptosis. Unlike apoptotic PCD, the role of ceramide and sphingosine-1-phosphate (S1P) in amino acids deprivation (AA(-))-induced autophagic PCD remains unclear. So, in this study, we examined the role of ceramide and S1P on the induction of autophagy and autophagic PCD. *Methods.* Human leukemia HL-60 cells were cultured in RPMI 1640 medium containing heat- inactivated 10% fetal bovine serum and transferred to AA(-) medium for induction of autophagy. Autophagy was assessed by the autofluorescent drug monodansylcadaverine (MDC), electronmicroscopy and cleavage of MAP-LC3 from 18 to 16kD. Apoptosis was judged by nuclear DAPI(4',6-diamidine-2-phenylindole dihydrochloride) staining. mTOR activation was assessed by *in vitro* kinase assay based on the levels of phosphorylation of 4E-BP1 and p70S6 with immunoprecipitated mTOR protein. The expression plasmid constructs used were the constructs for constitutively activated mTOR kinase and kinase-dead mTOR kinase, which have been previously described. HL-60 cells were transiently transfected by the electroporation method using NucleofectorTM kit (Amaxa Biosystems). *Results.* The generation of intracellular ceramide precedes AA(-)-induced autophagy and subsequent PCD in a caspase-3-independent manner in human leukemia HL-60 cells. S1P inhibits AA(-)- or N-acetylsphingosine (C2-ceramide)-induced autophagy through activation of mTOR, as judged by phosphorylation of 4E-BP1 and p70S6 kinase. In contrast, C2-ceramide overcomes S1P-inhibited induction of autophagy by inhibiting mTOR. Genetically overexpressed mTOR inhibits AA(-)- or C2-ceramide-induced increase of autophagy with activation of MAP-LC3, a mammalian homologue of yeast Apg8/Aut7, whereas overexpression of kinase-dead mTOR blocks the inhibitory effects of S1P on induction of autophagy by inhibition of MAP-LC3 activation. *Conclusions.* We here show that ceramide and S1P play an exclusive role on the induction of autophagy and autophagic PCD through the regulation of mTOR-dependent MAP-LC3.

0326

THE MACHANISMS UNDERLYING THE CYTOTOXIC EFFECT OF CDK INHIBITOR (ROSCOVITINE) ON LEUKEMIC CELL LINES

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Background. Roscovitine is a 2,6,9- trisubstituted aminopurine analogue that compete with ATP for binding to the active site on Cyclin-dependent kinases (CDKs). It inhibits CDK2/cyclinE, CDK7/cyclinH and CDK9/cyclinT. The cytotoxic effect of roscovitine and its analogues has been reported in several cancer cell lines *in vitro* and in animal models of cancer xenografts *in vivo*. The phase II clinical trails in lung and breast cancer and phase I trail in glomerulonephritis are currently ongoing. *Aim.* We have studied the mechanisms of roscovitine-induced cytotoxicity and cell death in leukemic cell lines HL60 (myeloid), Jurkat (lymphoblastic) and K562 (CML). *Methods.* HL60, Jurkat and K562 cells were cultured in RPMI1640 supplemented with 10% FBS. Cells were treated with Roscovitine in concentrations of 5 μM, 25 μM and 50 μM up to 48 hours. The cells were examined for viability using trypan blue exclusion assay, proliferation using 3H-thymidine incorporation assay, apoptosis using morphological criteria in Giemsa staining, cell cycle using propidium iodide and flow cytometry. Specific proteins were detected by Western blotting. *Results.* Cytotoxic effect of roscovitine expressed as decrease in viability and proliferation was concentration- and time dependent in HL60 and Jurkat cells. In contrast, no remarkable effect on K562 cells was observed. Apoptotic morphology was firstly observed 3h after the treatment with roscovitine and markedly increased 6h in HL60 and Jurkat cells, but not in K562 cells. In HL60 and Jurkat cells, the cell cycle analysis has shown an increase in sub-G1 cells at 6 hours with maximum at 24h without preceding cell cycle arrest. In K562 cells sub-G1 peak increased subsequently to G2/M arrest. In HL60 cells, cleaved fragment of caspase 2 was found from 6 hours of incubation with Roscovitine.

Activated fragments of caspases 3, 7 and 9 were observed at the same time point. PolyADP-ribose polymerase (PARP) was cleaved to 89kDA, confirming caspase-3 activation. In the mitochondrial pathway, Bcl-2 was cleaved to 23kDa and release of cytochrome c and AIF were observed. Activated fragment of caspase 8 was observed at 24 hours in Roscovitine 50uM. In Jurkat cells, caspase 2 was cleaved later than in HL60 (24 hours), while caspase 8 at 6 hours. Caspase 3, 7, and 9 were cleaved similarly as in HL60 cells. Release of cytochrome c and AIF from mitochondria at 6 hours was detected. However, Bcl-2 was not activated. In K562, no caspase activation was detected at studied time points. *Conclusion.* Roscovitine has shown a potent cytotoxic effect in both HL60 and Jurkat cells, whereas K562 has been resistant. Caspase 2 is involved in DNA damage and cytochrome c release in HL60. Apoptosis is induced by caspase 8 activation and mediated by mitochondrial pathway in Jurkat cell line. K562 cells are resistant to Roscovitine that maybe due to Bcr/Abl gene and loss of p53 function .

0327

THE BIOLOGIC SIGNIFICANCE OF CD40/CD40-LIGAND AND FAS/FAS LIGAND INTERACTIONS ON HAEMOPOIETIC PROGENITOR CELLS

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Background. Members of the tumor necrosis factor α (TNFα) family and their receptors (TNF-Receptors, TNFR), such as Fas and Fas-Ligand (FasL), have been implicated in the apoptotic depletion of the CD34+ haemopoietic progenitor cells. The molecules CD40/CD40L also belong to the TNFR/TNF family, however, their role in physiology and/or the pathophysiology of haemopoiesis is entirely unknown. *Aims.* To investigate the expression of CD40/CD40L molecules and their biologic significance on the haemopoietic progenitor cells. *Methods.* The human-derived CD34 positive myelogenous leukemia cell-line KG-1 has been used for this study. The expression of CD40 and Fas molecules on the KG-1 cells was evaluated using flow-cytometry under steady state conditions and following 72-hour incubation with different concentrations of recombinant human TNFa (rhTNFα). To probe the function of CD40 and Fas on the KG-1 cells, we investigated the expression of Fas and CD40, respectively, as well as the FRI and TNFR2 following 72-hour incubation with a combination of rhTNFa and rhCD40L or FasL, using flow-cytometry and semi-quantitative RT-PCR. The proportion of the apoptotic cells in the above conditions with or without the addition of rhFasL or rhCD40L respectively was studied, by flow-cytometry and the use of 7-aminoactinomycin-D (7-AAD) stain. *Results.* The KG-1 cells do not express CD40 and Fas under steady state conditions. However, the incubation of these cells with rhTNFα, upregulates the expression of the above molecules, in a dose-dependent manner (p<0.05 and p<0.05, respectively). The induction of CD40 on KG-1 cells, following incubation with rhTNFa, and its activation with rhCD40L, upregulates Fas (p<0.05) and TNFRI (p<0.05) expression, while downregulates the expression of TNFR2 (p<0.05), in mRNA as well as protein level. Similarly, the induction of Fas on KG-1 cells, following incubation with rhTNFa, and its activation with rhFasL, induces TNFRI (p<0.05) expression, while downregulates the expression of TNFR2 (p<0.05) and CD40 (p<0.05) in mRNA as well as protein level. Furthermore, the above induction and activation of CD40 on KG-1 cells, results in a significant increase in the proportion of apoptotic cells (34.2% ± 9.9%) compared to the proportion of apoptotic cells in the presence of rhTNFa alone (29.5% ± 9.9%; p<0.05). The presence of rhFasL increases further the proportion of apoptotic cells (62.4% ± 23.1%, p<0,05). *Summary-Conclusions.* The TNFR family member CD40 is not expressed under normal conditions on the CD34+ KG-1 cells. Its expression, however, is remarkably induced by TNFα. The activation of CD40 induces apoptosis of the cells and this effect is mainly mediated indirectly by up-regulating Fas and TNFRI and reinforcing therefore the apoptotic effect of the Fas/FasL and TNFα/TNFRI system on KG-1 cells. The interaction of CD40/CD40L with other TNF/TNFR family members may represent a contributing mechanism for the apoptotic depletion of CD34+ haemopoietic progenitor cells characterizing certain TNFα-associated bone marrow failure syndromes.

0328

METHYLATION-ASSOCIATED TRANSCRIPTIONAL SILENCING OF THE C/EBP α GENE IN ACUTE MYELOGENOUS LEUKEMIA

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Background. A regulatory network including various transcription factors controls the differentiation of hematopoietic stem cells and progenitor cells. The CCAAT/enhancer binding protein α (C/EBPα) is a transcription factor implicated in the regulation of myelopoiesis that plays an important role in the coordination of cellular differentiation with growth arrest. Specific point mutations of C/EBPα have been reported in acute myelogenous leukemia (AML). Mutated forms of C/EBPα may impair granulocytic differentiation and thus contribute to leukemogenesis. *Aims.* Aberrant CpG island methylation in association with transcriptional silencing has been recognized to act as an alternative to mutations and deletions to disrupt tumor suppressor gene function. A large number of genes involved in fundamental cellular pathways have been shown to be affected by this epigenetic phenomenon. In this study, we investigated the possible role of CpG island hypermethylation in the transcriptional regulation of the C/EBPα gene in AML. *Methods.* Aberrant methylation of C/EBPα in hematopoietic cell lines and patient samples was assessed by methylation specific PCR (MSP). Methylation patterns in cell lines were further analyzed in detail by bisulfite sequencing. Expression of C/EBPα was determined by real time reverse transcription PCR. *Results.* In hematopoietic tumor cell lines, aberrant methylation of the C/EBPα promoter region was associated with transcriptional silencing. Treatment of cell lines, which carry a hypermethylated C/EBPα gene, with the demethylating agent 5-aza-2′-deoxycytidine resulted in C/EBPα reexpression. In the cell lines L540 and Raji, bisulfite sequencing of individual alleles revealed dense methylation throughout the region around the transcription start site, while HL-60 cells were almost completely unmethylated. The analysis of diagnostic bone marrow and blood specimens from adult patients with AML by MSP showed aberrant methylation of the C/EBPα promoter region in 12/69 (17.4%) samples. Hypermethylation of C/EBPα in AML could be detected in all cytogenetic risk groups, but was restricted to the French-American-British (FAB) subtypes M1, M2, M4 and M5. There was a trend towards a better overall survival in AML cases with C/EBPα hypermethylation. *Summary*. These data indicate that hypermethylation of the transcription factor C/EBPα is a common epigenetic event in adult AML. Hypermethylation-associated silencing of C/EBPα may, in addition to genetic aberrations, interfere with the cellular differentiation process and thus contribute to the malignant phenotype. The exploration of our growing knowledge about epigenetic aberrations in leukemogenesis may help develop novel strategies in diagnosis and treatment of AML for the future.

0329

ROLE OF SIGNAL TRANSDUCTION PATHWAYS AND THE MICROENVIRONMENT IN THE EMERGENCE OF MINIMAL RESIDUAL DISEASE IN ACUTE MYELOID LEUKEMIA

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Background. Relapse is common in patients with acute myeloid leukemia (AML) due to the emergence and outgrowth of minimal residual disease (MRD). High frequency of flow cytometric (FACS) detected MRD identifies patients with high risk of relapse (Feller *et al.,* Leukemia 2004; 18: 1380). New treatment strategies are therefore needed to effectively eradicate these MRD cells in order to improve survival. *Aims.* Our research focuses on how aberrant signal transduction, e.g. constitutive phospho-AKT (pAKT) and phospho-ERK (pERK) expression and Nuclear Factor kappa B (NFkB) activity, all in interaction with the bone marrow microenvironment (BM-ME) contribute to the emergence, persistence and outgrowth of MRD and thereby effect prognosis of the patient. *Methods.* A sensitive and reproducible FACS assay was developed for the quantification of phosphorylated protein expression in AML. *Results.* Good correlations were found between the FACS assay and Western Blot- and ELISA techniques in both cell lines and patient samples. Specificity of the signals was proven using the inhibitors LY294002, for PI3K-dependent AKT phosphorylation, U0126 for MAPK dependent ERK phosphorylation, and MG132 a protoasome inhibitor for NFkB activity. Using a NFkB activity ELISA both a cell line (HL60) and patient samples (n=3) showed NFkB activity that was upregulated by adherence to

fibronectin to simulate the BM-ME: factor 1.6 in HL60 and 1.5 in 3/4 patient samples, with no or less upregulation in non-adherent cells. Response of pAKT, pERK and pNFkB in reaction to fibronectin binding is under investigation using firstly Western Blot but eventually using our FACS assay after optimalisation for the BM-ME conditions. Subsequently, the FACS assay was adapted to study AML subsets, in particular stem cells (CD34+CD38-) and MRD cells. pAKT, pERK and pNFkB expression could be shown in the CD34+CD38- stem cells. Also the expression of pAKT, pERK and pNFkB in subpopulations with aberrant immunophenotypes (e.g. CD34+CD7+, CD34+CD56+) enables the comparison with signal transduction in MRD cells identified by these aberrancies. *Summary/Conclusions.* AKT, ERK and NFkB signaling can now be studied in subpopulations highly relevant for clinical outcome, i.e. stem cells, MRD cells and MRD stem cells. We have shown how to detect stem cells under MRD conditions (van Rhenen *et al.* Blood 2005; 106: 4, abstract van Rhenen *et al.* this conference). In particular changes of signaling in the course of disease, as may be inferred from reported FLT3 ITD changes from diagnosis to relapse, urge the possibility to study signal transduction under MRD conditions. This and the study of the interactions between leukemic cells and the microenvironment contributing to persistence and outgrowth of MRD might ultimately guide the development of new treatment strategies, directed at the MRD (stem) cell and/or the microenvironment.

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0330

TRANSIENT POST-TRANSLATIONAL UPREGULATION OF TELOMERASE ACTIVITY DURING MEGAKARYOCYTIC DIFFERENTIATION

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Background. Telomerase is a ribonucleoprotein reverse transcriptase that adds hexameric repetitive sequences (TTAGGG) to the ends of chromosomes. Telomerase plays a key role in maintaining telomere length and in replicative senescence. Telomerase is active in immature somatic cells and is suppressed in differentiated cells, but the mechanism by which telomerase activity is regulated in relation to cell differentiation remains unclear. Several regulatory of mechanisms for telomerase have been reported, including 1) transcriptional, 2) translational, and 3) post-translational mechanisms, suggesting that the regulation of telomerase activity is a complex process. *Aims.* To determine the mechanisms modulating telomerase activity during differentiation in various lineages of hematopoietic cells. *Methods.* A human chronic myelogenous leukemia cell line (K562) was induced to differentiate into megakaryocytes by exposure to TPA, and into erythroid cells by exposure to STI571. A human acute myeloblastic leukemia cell line (HL60) was induced to differentiate into monocytes by exposure to TPA. To assess the effect of PKC inhibitors during megakaryocytic differentiation, K562 cells were preincubated with Bisindolylmaleimide or Rottlerin, and then TPA was added before further incubation. Telomerase activity, the expression of human telomerase reverse transcriptase (hTERT) protein, mRNA, and functional binding transcription factors within the telomerase promoter region were examined. Cells were separated into cytoplasmic and nuclear fractions to examine the localization of telomerase. *Results.* TPA induced a transient increase of telomerase activity during the megakaryocytic differentiation of K562 cells, while expression of hTERT decreased gradually throughout differentiation. The transient increase of telomerase was mainly observed in the nuclear fraction rather than the cytoplasmic fraction. Pretreatment of K562 cells with a PKC inhibitor blocked both megakaryocytic differentiation and the transient increase of telomerase activity. In addition, a dose-dependent increase of telomerase activity after exposure to recombinant PKC was observed. To further assess the transcriptional control mechanism of telomerase, a chromatin immunoprecipitation (ChIP) assay was performed. STAT3 (which was bound to the hTERT promoter) became dissociated from the promoter during megakaryocytic differentiation, while Sp1 remained stable during differentiation. *Conclusions.* A transient increase of nuclear telomerase activity was detected during megakaryocytic differentiation stimulated by TPA, and this increase was suppressed by PKC inhibitors. In addition, telomerase activity was dose-dependently increased by recombinant PKC. These results suggest that PKC is one of the post-translational regulators of telomerase activity during the megakaryocytic differentiation of K562 cells. Megakaryocytes are unique hematopoietic cells that undergo DNA replication during differentiation into mature polyploid cells. This may mean that post-translational activation of telomerase is necessary for the immediate stabilization of replicated chromosomes before the *de novo* synthesis of telomeres commences. On the other hand, STAT3 was suggested to be one of the transcription factors regulating telomerase during megakaryocytic differentiation. These results indicate that telomerase activity during megakaryocytic differentiation stimulated by TPA is regulated at least by two mechanisms, with one being transcriptional and the other being post-translational.

0331

PAX5/TEL CAUSES DOWN MODULATION OF CD19 IN PRE B CELLS

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Background. we previously cloned the PAX5/TEL chimeric gene, originated from the translocation t(9;12)(q11;p13) in an ALL patient. Recent data indicate that the PAX5/TEL fusion defines the cytogenetic entity dic(9;12)(p13;p13), a recurrent chromosome abnormality that accounts for about to 1% of childhood ALL, almost exclusively B-progenitor ALL. PAX5/TEL is likely to be an aberrant transcription factor, resulting from joining the 5' region of PAX5 (a transcription factor essential for B cell development) to the 3' region of TEL/ETV6 (Ets-family DNA binding domain). Aim of the study was to investigate the functions of the PAX5/TEL chimeric protein in preBI cells. *Methods.* we have cloned the FLAG-full length chimeric PAX5/TEL cDNA in the retroviral vector pMSCV-IRES-GFP (MigR1). Murine PAX5 -/- preBI cells and wild type preBI cells were transduced with the retroviral construct to analyze cell proliferation, differentiation and growth-dependence on IL-7. Both PAX5 -/- preB I cells and wild type preBI cells were cultured on OP9 and DL1-OP9 stroma cells. *Results.* wild type preBI cells, transduced with pMSCV-PAX5/TEL-IRES-GFP vector, showed down modulation of CD19 when cultured on OP9 stroma in presence of IL-7. Semiquantitative RT-PCR didn't show any difference in transcription of PAX5 target genes such as BLNK, MB-1, M-CSFR. PAX5TEL-preBI cells cultured on DL1-OP9 showed a different phenotype, with up-regulation of c-KIT and down-regulation of CD44. PAX5-/- preBI cells infected with PAX5TEL and grown on OP9 were CD19 negative even in the presence of PAX5TEL; in absence of IL-7 they died following the same kinetic of the control cells. By semiquantitative RT-PCR, we didn't detect mRNAs of CD19 and no difference in BLNK, MB-1 and M-CSFR mRNA level was found. On DL1-OP9, PAX5TEL cells were able to differentiate maintaining the developmental plasticity of PAX5 -/- pre BI cells. *Conclusions.* preliminary results showed a role of PAX5TEL as a transcriptional suppressor, down regulating CD19 expression, thus suggesting a function on B cell differentiation. PAX5TEL cannot replace PAX5 functions in PAX5-/- cells. Further analysis are needed to better evaluate the role of PAX5/TEL protein, both *in vivo* and *in vitro* models.

0332

OVEREXPRESSION OF 14-3-3 SIGMA IS ASSOCIATED WITH TYROSINE KINASE ACTIVITY OF P210 BCR-ABL FUSION PROTEIN OF CHRONIC MYELOID LEUKEMIA

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The 14-3-3 proteins are a family of phosphoserine/threonine-binding molecules and critical mediators of intracellular signaling pathways, including those controlling proliferation, cell cycle checkpoint activation and survival. In particular, upon c-Jun NH2-terminal kinase (JNK)-mediated phosphorylation in response to stress they release c-abl tyrosine kinase and let its nuclear import, the prerequisite fot its pro-apoptotic and growth arrest function. Here we show that constitutive tyrosine kinase (TK) activity of p210 bcr-abl protein, the molecular hallmark and causative event of Chronic Myeloid leukemia (CML) is associated with overexpression of 14-3-3 sigma. In 32D cell clones transducing a temperature-sensitive bcr-abl construct the levels of 14-3-3 transcript and protein were increased under permissive culture conditions for p210 TK and significantly reduced by p210 TK inhibition by the TK inhibitor Imatinib mesylate (IM). Moreover, in K562 CML cell line we observed the hyperacetylation of a discrete region of 14-3-3 sigma promoter that corresponds to -8245 to -8508, that was significantly reduced since 4th hour of exposure to IM. Conversely, the methylation status at a CpG-rich area of 14-3-3 sigma coding region including the transcription start site (-220 to + 116) was not conditional upon p210 TK. Our results support that p210 TK influences 14-3-3 sigma trascription rate by interacting with epigenetic mechanisms that control chromatin accessibility. Interestingly, in K562 cell line IM resistance was associated with a further increse of 14-3-3 sigma expression and higher hyperacetylation at its promoter, supporting a putative role of this scaffolding protein in clonal evolution of CML progenitors

towards drug resistance. Further studies are presently in progress to elucidate mechanisms relevant for 14-3-3 overexpression and enhanced binding properties, whether they may be targeted by drug combinations that have been advanced for clinical trials.

0333

THE INVOLVEMENT OF C:18 CERAMIDE AND HUMAN LONGEVITY ASSURANCE GENES IN IMATINIB INDUCED APOPTOSIS

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Background. Ceramides have essential roles in many aspects of cell metabolism, from inflammatory responses through the regulation of cancer-cell growth, cell proliferation, apoptosis, cell migration and senescence. Many cytokines, anticancer drugs and other stress-causing agonists result in increases in endogenous ceramide levels through *de novo* synthesis and/or the hydrolysis of sphingomyelin. Since human longevity assurance genes (LASS) are responsible for the de-novo synthesis of ceramides, the expression levels of LASS genes are important in stress induced apoptosis. *Aims.* Ceramide metabolism in imatinib induced apoptosis in hematological malignancies was examined in this study. Sensitive and resistant chronic myeloid leukemia (CML) cells, K562 were used as a model system to investigate the changes in ceramide metabolism upon imatinib treatment. *Methods.* The Ph^+ human K562 cells were exposed to step-wise increasing concentrations of imatinib. Subpopulations of cells those were able to grow in the presence of 0.2 and 1 μM imatinib, were then selected, and referred to as K562/IMA-0.2 and K562/IMA'1, respectively. Caspase-3 activity was determined using the caspase-3 colorimetric assay. The mitochondrial membrane potential (MMP) was measured using a JC-1 MMP detection kit. The cellular levels of endogenous ceramides were measured using high performance liquid chromatography/mass spectrometry (LC/MS). Plasmid and siRNA transfection of K562 cells were conducted using an Effectine and DharmaFECT™ siRNA transfection reagent, respectively. *Results.* Measurement of endogenous ceramide levels by LC/MS showed that treatment with imatinib increased the generation of ceramide, particularly C:18-ceramide, significantly in a time-dependent manner in parental sensitive cells, whereas in resistant cells, there was no significant changes in its levels in response to imatinib at 48 hr. Partial inhibition of human longevity assurance gene 1 (hLASS1) by small interfering RNA (siRNA), which prevented C:18-ceramide generation, partially inhibited imatinib-induced cell death, as detected by activation of pro-caspase-3, and loss of mitochondrial membrane potential, in sensitive K562 cells. In reciprocal experiments, overexpression of hLASS1 caused a marked increase in imatinib-induced C:18-ceramide generation and apoptosis in resistant K562/IMA-0.2 and K562/IMA-1 cells. Interestingly, analysis of mRNA levels of hLASS1, for the generation of C:18-ceramide did not show any significant differences in these resistant cells when compared to controls, suggesting that accumulation and/or metabolism, but not rate of synthesis, might be altered in imatinib-resistant cells. *Summary/Conclusions.* These data suggest that increased ceramide generation and/or accumulation might be involved in mediating imatinib-induced apoptosis, and that defects in C:18-ceramide accumulation and/or metabolism might play a role in a decrease in imatinib-induced apoptosis, thus results in resistance to therapy.

0334

SURVIVIN AND BCL2 EXPRESSION IN CD30-POSITIVE LYMPHOPROLIFERATIVE DISORDERS OF THE SKIN COMPARED TO SYSTEMIC ANAPLASTIC LARGE CELL LYMPHOMAS: AN IMMUNOHISTOCHEMICAL STUDY OF 28 CASES

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Backgrounds. Cutaneous CD30-positive lymphoproliferative disorders (LPDs) are a spectrum of indolent diseases ranging from lymphomatoid papulosis (LyP) to primary cutaneous anaplastic large lymphoma (C-ALCL). Apoptosis has been investigated in systemic anaplastic large cell lymphomas (ALCL), which are potentially aggressive, but has not been elucidated in cutaneous CD30-positive LPDs. *Aims.* We investigated the expression of two inhibitors of apoptosis, survivin and BCL-2 protein, in a series of cutaneous primitive CD30-positive LPDs and systemic ALCL, in order to highlight differences that may eventually help in the differential diagnosis and related to different biological behaviour. *Materials and Methods.* We retrieved from our files 28 cases of T-cell CD30-positive LPDs diagnosed between 1995 and 2003 with the clinical history: 10 cutaneous CD30-positive LPDs (5 LyP, 5 C-ALC) and 18 systemic ALCL. Immunohistochemical analysis was performed with antibodies against ALK1 protein, survivin and BCL-2 protein, on tissue sections with the DAKO Envision system. RT-PCR studies for ALK and ALK/NPM were performed on RNA extracted from paraffin blocks of all 28 cases. *Results.* All the cutaneous CD30+ LPDs were negative for ALK by immunostaining and RT-PCR. Among systemic ALCL cases, 7 were ALK-positive and 11 were negative: all the positive cases showed a 366 bp ALK transcript by RT-PCR and the specific NPM/ALK fusion transcript of 98 bp, ruling out the presence of a different rearrangement. All the 28 cases examined showed a clear cytoplasmic positivity for survivin, independently from their clinicopathological group. Five cases of systemic ALCL, which were all ALK negative, showed in addition a nuclear dot-like immunoreactivity for survivin. Nuclear expression of survivin was not observed in the other groups (chi2: p=0.045). Protein BCL-2 cytoplasmic expression was found in 10 cases; systemic ALK-positive ALCL show a lower frequency of BCL-2 expression (chi2: p=0.045). *Conclusions.* Our result showed that LyP and C-ALCL share a heterogeneous expression of cytoplasmic survivin and BCL-2, similarly to systemic CD30-positive lymphomas, suggesting that survivin might be expressed also in indolent and potentially regressing lesions and is not considerable an absolute marker of malignancy. Survivin has been indeed demonstrated in many nonneoplastic cells of non-lymphoid nature. BCL-2 was also expressed in half of our cases of LyP and PC-ALCL, similarly to systemic ALCL, suggesting that either BCL-2 nor cytoplasmic survivin expression does not help in distinguishing in the spectrum of cutaneous CD30-positive LPDs nor between cutaneous and systemic diseases. It might be postulated that apoptosis is still potentially inducible in these BCL-2 and survivin-expressing cells because these lesions have the potential to undergo spontaneous regression. Alternative mechanisms including the immune control mediated by activated cytotoxic lymphocytes (CTL) can play a major role in these indolent diseases as postulated in systemic disorders. Our data confirm that BCL-2 is less frequently expressed in ALK-positive than in ALK-negative systemic ALCL cases. The most interesting and unexpected feature was the observation that 45% of our systemic ALK-negative ALCL cases showed nuclear survivin immunostaining, in contrast with others who found survivin exclusively located in the cytoplasm by immunohistochemistry and by Western blotting.

0335

TARGETING IAPS OVERCOMES APOPTOSIS RESISTANCE OF PANCREATIC CARCINOMA CELLS AND SUPPRESSES TUMOR GROWTH AND INVASION *IN VIVO*

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Pancreatic cancer is one of the leading causes of cancer-related death due to its resistance towards conventional therapies. To improve cancer therapy, it is crucial to better understand the molecular mechanisms underlying apoptosis resistance of pancreatic cancer. Here, we identify X-linked inhibitor of apoptosis (XIAP) as a key determinant of apoptosis resistance of pancreatic carcinoma cells. XIAP was expressed at high levels in the majority of pancreatic carcinoma cell lines and primary tumor samples. Stable downregulation of XIAP by RNA interference significantly reduced viability and enhanced TRAIL-induced apoptosis in pancreatic carcinoma cells. Importantly, knockdown of XIAP also strongly inhibited clonogenicity of pancreatic cancer cells treated with TRAIL indicating that XIAP promotes clonogenic survival. Also, downregulation of XIAP significantly increased CD95- or γ-irradiation-induced apoptosis, whereas it had no effect on 5-fluorouracil, etoposide or gemcitabine-induced apoptosis. Analysis of apoptosis signaling pathways revealed that knockdown of XIAP resulted in enhanced activation and enzymatic activity of caspase-3, -9, -2 and -8. Interestingly, downregulation of XIAP also led to enhanced drop of mitochondrial membrane potential and increased cytochrome c release after stimulation with TRAIL, indicating that XIAP functions upstream of mitochondria in TRAIL-induced apoptosis. In support of this notion, inhibition of caspase-3 completely inhibited drop of mitochondrial membrane potential in TRAIL-treated pancreatic carcinoma cells, in which XIAP was knocked down. Most importantly, knockdown of XIAP profoundly inhibited tumor growth and invasion of pancreatic carcinoma cells *in vivo*. Similarly, inhibition of XIAP by small molecule antagonists sensi-

tized pancreatic cancer cells to TRAIL-, CD95- or γ-irradiation-induced apoptosis. By demonstrating that targeting IAPs significantly enhanced death receptor or γ-irradiation-induced apoptosis and also suppressed tumor growth and invasion of pancreatic carcinoma cells *in vivo*, our findings indicate that targeting IAPs represents a novel, promising strategy to overcome apoptosis resistance of pancreatic cancer, which has important clinical implications.

0336

TRAIL RECEPTORS IN B-CLL PATIENTS INDEPENDENTLY OF ZAP 70 EXPRESSION

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B-cell lymphocytic leukaemia (B-CLL) is an incurable disease with an indolent course characterized by a low growth fraction and a progressive expansion of a subpopulation of monoclonal B lymphocytes (LLC-B cells) which are functionally inactive and resistant to apoptotic cell death. The most specific way to induce apoptosis is stimulation of the different types of death receptors with their specific ligands. TRAIL (Tumour necrosis factor (TNF) - related apoptosis inducing ligand) receptors are members of TNF receptor family, are widely expressed on the surface of many different cell types and induce selective apoptosis of cancer cells but not of normal cells. They can be activated by TRAIL. This ligand can interact with 5 receptors, TRAIL-R1 (DR4), TRAIL-R2 (DR5), TRAIL-R3 (DcR1), TRAIL-R4 (DcR2) e Osteoprotogerin (OPG). The stimulation of the death receptors, DR4 and DR5 can induce apoptosis. However, the expression of DcR1, DcR2 and OPG receptors may counteract TRAIL apoptotic effect. Thus, the expression of these types of receptors can determined the failure to undergo apoptosis in B-CLL and may have direct implications for B-CLL therapy. The aim of this study is to evaluate the expression of TRAIL receptors, DR4, DR5, DcR1 and DcR2 in B Chronic Lymphocytic Leukaemia patients and its correlation with the expression of the prognostic markers ZAP 70 and CD11c. For this purpose directly conjugated monoclonal antibodies to CD5 and CD19 was used to identify LLC-B, T and B cells obtained from 22 patients with B-CLL. These patients were divided according the expression of the ZAP 70 protein (6 ZAP 70+; 6 ZAP 70- and 10 non determined) and the _-integrin CD11c (10 CD11c+ and 6 CD11c-). The surface expression of TRAIL receptors, DR4, DR5, DcR1 and DcR2, were analysed by flow cytometry, using specific monoclonal antibodies. The results are expressed in Mean Intensity Fluorescence (MIF). We observe in LLC-B cells lower levels of proapoptotic TRAIL receptors, namely DR5 (18.6±8.1 MIF) compared with normal B cells (35.7 _ 20.5) and higher expression of the antiapoptotic DcR1 receptor witch may contribute to resistance of LLC-B cells to death receptor-mediated apoptosis. Preliminary results didn't show any correlation between TRAIL receptors expression and the above mentioned prognostic markers. However a higher number of patients must be included in the study in order to clarify our results. *Aknowledgments.* Prof. Catarina Oliveira and Dra Luísa Pais, Directors of Biochemistry Institute, Faculty of Medicine, University of Coimbra, and Histocompatibility Center of Coimbra, respectively

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P38-MEDIATED ACTIVATION OF CASPASES IN C2-CERAMIDE-INDUCED APOPTOSIS OF MOUSE HEMATOPOIETIC CELLS

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Cell-permeable C2-ceramide induces apoptosis in various types of cell. Here, we have studied the effects of C2-ceramide on mouse bone marrow cells containing primitive hematopoietic progenitors (PHP), as little is known about the signaling pathway in the apoptosis in PHP induced by C2-ceramide. The C2-ceramide was found to induce apoptosis in a time dependent manner, as defined morphologically by nuclear condensation and fragmentation visualized with propidium iodide staining and by a positive annexin V (AV) and by a reduced colony forming ability of the bone marrow cells of mouse. C2-ceramide suppressed colony growth derived from mouse Day-2 post 5-FU marrow cells (5-FU marrow cells) in a dose dependent manner. Incubation of 5-FU marrow cells with 15 μM of C2-ceramide for three hours gave 41.7±17.2% of mean%control of colonies. Further, an extended study using more PHP-enriched lineage marker-negative cells (Lin- cells), which were isolated from mononuclear 5-FU marrow cells, revealed that C2-ceramide completely suppressed colony formation. To obtain direct evidence of induction of apoptosis in Lin- cells, we detected that about 90% of AV- cells were changed into AV+ cells in Lin- cells by incubation with C2-ceramide, suggesting PHP were actually induced into apoptosis. Next, we studied the effects of C2-ceramide on the intracellular activation of caspases in PHP, using the cell-permeable fluorescence-labeled substrates of several caspases and the colorimetric caspase activation assay kits. C2-ceramide treatment showed fluorescence and colorimetric caspases activation of 5-FU marrow cells, which increased in intensity within one hour. However, selective caspase inhibitors inhibited the fluorescence and colorimetric release of each caspase substrates, indicating the specific involvement of caspases in the C2-ceramide-induced apoptosis of PHP. Further, the selective inhibitors for caspases also prevented nuclear condensation and fragmentation and suppression of colony formation of 5-FU marrow cells. Based on the effect of these caspase inhibitors on the activation of each fluorogenic caspase substrate, C2-ceramide is believed to activate LEHD-, DEVD-, and VEID-cleaving caspases such as caspase-9, -3, and -6, respectively, in that order. Upstream of the caspases, C2-ceramide activated p38 and the selective p38 inhibitor SB203580, thus reversed the activation of these caspases that had been induced by C2-ceramide, resulting in a significant recovery from apoptosis. On the other hand, IETD-cleaving caspase such as caspase-8 was not activated by C2-ceramide. These results suggest that C2-ceramide initiates apoptosis in PHP via activation of the caspase-9-dependent caspase cascade mediated by p38.

0338

CERAMIDE GLYCOSYLATION BY GLUCOSYL CERAMIDE SYNTHASE INHIBITS THE APOPTOTIC EFFECT OF IMATINIB ON HUMAN K562 AND MEG-01 CELLS

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Background. Glucosyl ceramide (Glc-Cer) has recently been shown to be associated with resistance to chemotherapy. Alterations in ceramide metabolism, whereby pro-apoptotic ceramide is converted to its non-cytotoxic Glc-Cer metabolit resulting in drug resistance. Accumulation of Glc-Cer is a characteristic of some MDR cancer cells and tumors derived from patients who are less responsive to chemotherapy. Several tumor cell lines and clinical samples have been shown to overexpress the glucosylceramide synthase (GCS) enzyme, which transfers glucose from UDP-glucose to ceramide and produces Glu-Cer. *Aims.* The effects of GCS in imatinib resistance in human chronic myeloid leukemia (CML) cells were investigated. The cells were exposed to a combination of imatinib and GCS inhibitors, D-threo-1-phenyl-2-decanoylamino-3-morpholinopropan-1-ol (PDMP) or N-(n-Nonyl)deoxygalactonojirimiycin (C9DGJ) to determine if resistant cells could be sensitized. *Methods.* The Ph^+ human K562 and Meg-01 cells were exposed to step-wise increasing concentrations of imatinib. Subpopulations of cells that were able to grow in the presence of 0.2 and 1 μM imatinib, were then selected, and referred to as K562/ or Meg-01/IMA-0.2 and Meg-01/IMA'1, respectively. Plasmid transfection of K562 cells were conducted using an Effectine transfection reagent. The expression pattern of GCS was detected by RT-PCR and western blotting. The IC50 values were determined from cell survival plots obtained by MTT. Cell cycle profiles of cells were analyzed by flow cytometry. The cellular levels of endogenous ceramides were measured using high performance liquid chromatography/mass spectrometry (LC/MS). *Results.* Measurement of the levels of GCS by RT-PCR and Western blotting demonstrated that the expression of GCS was increased in both K562/IMA-1 and Meg-01/IMA-1 cells as compared to parental sensitive cells. The possible role of GCS in resistance to imatinib was further examined by transfection and the overexpression of GCS gene in K562 and Meg-01 cells. GCS overexpression in sensitive cells resulted in an increase in imatinib-resistance. There was a significant increase of apoptosis by co-application of imatinib and GCS inhibitors (PDMP or C9DGJ) after 48 hr in both sensitive and resistant cells. There was also an up-regulation of ceramide levels measured using high performance liquid chromatography/mass spectrometry (LC/MS) by co-application of imatinib and GCS inhibitors (PDMP or C9DGJ) to K562 and Meg-01 cells. *Summary/Conclusions.* These results may suggest the involvement of GCS, by converting pro-apoptotic ceramide to Glu-Cer, in imatinib resistance. Besides, inhibiting the GCS activity by co-appli-

cation of PDMP and/or C9DGJ increased the sensitivity of CML cells to imatinib.

0339

NOSCAPINE INDUCES APOPTOSIS THROUGH ACTIVATION OF CASPASES AND MITOCHONDRIAL EVENTS IN P53-NULL MYELOBLASTIC LEUKEMIA CELL LINE K562

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Monitoring apoptosis is becoming increasingly important in finding new chemotherapeutic drug and their mechanism. Previously, the microtubule opium alkaloid noscapine was discovered as a microtubule destabilizing agent that arrests mammalian cells at mitosis and induces apoptosis. Because noscapine is water-soluble and absorbed after oral administration, and has little toxicity to normal tissue and no inhibition of immune responses, its chemotherapeutic potential in human cancer merits through evaluation. We selected drug resistant, P53-null myelogenous leukemia cells to monitor apoptosis and study of noscapine's mechanism. K562 cells showed delayed but effective response to noscapine treatment, and we could monitor apoptosis by the DNA fragmentation, PARP cleavage and increasing activity of caspase 2,3,6,9 with 20 μM noscapine after 24-48hr treatment. The increased Bax/Bcl-2 ratio more than three times with 20 μM noscapine in time dependent manner from 3-48 hr can prove some mitochondrial event in response to this drug. These results help to elucidate some critical points in noscapine mechanism as a good candidate for preventive and therapeutic application in chronic myeloid leukemia.

0340

ERYTHROID-SPECIFIC TRANSCRIPTIONAL REGULATION OF THE HUMAN PROTOPORPHYRINOGEN OXIDASE GENE IS MEDIATED BY TWO GATA-1 SITES IN EXON 1

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Background. Protoporphyrinogen oxidase (PPOX) catalyzes the six-electron oxidation of protoporphyrinogen IX to protoporphyrin IX. Like other heme biosynthetic proteins, PPOX is involved in synthesizing heme for red cells (erythroid-specific expression) and heme as a cofactor for the respiratory cytochromes (housekeeping expression). Whereas tissue specific regulation of other heme biosynthetic enzymes is extensively studied, there is little knowledge concerning transcriptional regulation of PPOX. *Aims.* The aim of this study was to investigate molecular mechanisms involved in the erythroid-specific regulation of PPOX. *Methods.* Functional studies were performed using transient transfection of PPOX promoter constructs in human K562 erythroleukemia cells. DNA-protein interaction at the GATA-1 sites in exon 1 of PPOX was studied using Electrophoretic Mobility Shift Assay's (EMSA) with nuclear extracts from K562 cells. *Results. In vitro* transfections studies revealed that reporter constructs containing exon 1 showed a 300% increase in promoter activity compared to constructs lacking this exon. Transfection experiments of wild-type and mutant reporter plasmids in K562 cells demonstrated that erythroid-specific transcriptional regulation of PPOX was mediated by two GATA-1 sites in exon 1. The highest level of transcription depended on the integrity of both sites. Electrophoretic mobility shift assay and supershift experiments using K562 nuclear extracts demonstrated that both GATA sites were able to bind GATA-1 *in vitro*. Exon 1 did not have any effect on PPOX promoter activity in human hepatoma HepG2 cells. In HeLa human cervical carcinoma cells, however, the presence of exon 1 decreased promoter activity. *Summary/Conclusions.* Exon 1 of the human PPOX gene contains two GATA-1 binding motifs, which both are required for erythroid-specific expression of PPOX and, in addition, bind GATA-1 *in vitro*. These results contribute to a better understanding of the molecular mechanisms involved in differential regulation of the human PPOX promoter in erythroid and non-erythroid cells.

0341

THE NOVEL GENE JUNE-1 IS DIFFERENTIALLY EXPRESSED DURING ERYTHROID DIFFERENTIATION AND IS EXPRESSED IN TUMOUR CELLS

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Background. The hormone erythropoietin (EPO) leads to red blood cell development after binding to EPO receptors (EPO-Rs) on erythroid progenitors. However, little is known about the genes that are regulated downstream of EPO. EPO-Rs have also been found on a variety of non-haematopoietic tissues, including some tumours; an important finding since rHuEPO is often used to treat anaemia in cancer patients. Using differential display PCR and RNAse protection assays several differentially expressed genes were identified after EPO stimulation in two mouse models of erythroid differentiation. One of these is a novel gene which we have named June-1. *Aims.* To characterize June-1 and identify its function. *Methods and Results.* The June-1 protein displays significant sequence similarity to only one other protein, PHF13, also of unknown function. We have identified homologues of June-1 in many vertebrates, but not in lower species, suggesting that it is a recently evolved gene. Across species there is a high degree of sequence conservation, suggesting that June-1 has an important function. This 5 exon gene (1.2 kb transcript and predicted 44 kDa protein) contains a nuclear localization domain, PHD domain (zinc finger), and potential SUMOylation site. This, along with further in silico analysis, suggest that June-1 may function as a transcription factor or be involved in chromatin remodelling. JUNE-1 has now been examined in a human model of erythroid differentiation (K562 cells), where it is upregulated, confirming that JUNE-1 has a role in erythropoiesis. Promoter analysis of this gene reveals a potential GATA binding site which may be important in erythroid function. To further elucidate the role of JUNE-1 as a possible transcription factor, GFP tagged JUNE-1 is being expressed in K562 cells to examine the subcellular localization of the protein at different developmental stages. Chromatin immunoprecipitation studies are underway in order to identify possible gene targets of JUNE-1. In addition, knockdown and overexpression studies are ongoing to determine the function of JUNE-1 in K562 cells and identify protein binding partners. Preliminary studies indicate that knockdown of JUNE-1 leads to a decrease in cellular proliferation, but does not affect the rate of differentiation RT-PCR and Q-PCR analyses indicate that June-1 is widely expressed across a variety of normal tissues, suggesting that it may have roles in addition to erythropoiesis. We have also shown expression of June-1 in a variety of tumour cells. Human JUNE-1 maps to chromosome 17p13, just distal to the p53 tumour suppressor gene; genetic alterations of this chromosomal region are frequently seen in human malignancies and several reports in the literature suggest that this region may contain an additional important tumour suppressor gene. We have identified a breast cancer cell line that is responsive to EPO and expresses JUNE-1 and are studying the role of JUNE-1 in these cells, including its role in the regulation of proliferation and signal transduction. *Summary.* June-1 is a novel gene identified downstream of EPO stimulation which appears to be involved in erythroid differentiation and may act as a transcription factor.

0342

CLINICAL AND PROGNOSTIC SIGNIFICANCE OF P53 GENE MUTATION IN ACUTE LEUKEMIA

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Back ground and aim of the work. P53 is a tumor suppressor gene, located at the chromosomal region 17p 13, consisting of 10 introns and 11 exons, of which exons 2 to 11 are transcribed. 3- Wild type (wt) P53 acts as tumor suppressor protein whereas mutant (mut) P53 may exhibit gain of function properties such as immortalization of primary tumor cells. The P53 protein plays a crucial role in maintaining genetic stability at the cellular level.This work was planned aiming to recognize the potential role of P53 in leukemogenesis and explain the correlation between mutations of P53 in acute leukemic patients and clinical subtypes, clinical behavior and prognosis of the disease.Subject and methods This study was carried on 38 patients with acute leukemia, twenty eight of them were newly diagnosed patients and ten patients were at relapse. Accordingly they were categorized into 3 groups:Group I (at diagnosis): This group was included 28 newly diagnosed patients with acute leukemia. They were classified into 15 cases with ALL and 13 cases with AML.Group II (after induction): this group was included 28 patients who were followed up after induction for 1, 6 and 12 months. Group III: This group included 10 relapsed patients with acute leukemia (sampling taken once at relapse). They were classified into 5 cases with ALL and 5 cases with AML. In additions 10 subject were selected as control group Patients and controls were subjected to the following laboratory investigation Complete blood picture, erythrocyte sedimentation rate,Liver function tests and s. creatinine, s. uric acid and serum L.D.H.Bone marrow aspiration smears, cytochemistry stains on blood or bone marrow smears:were helpful in distinguishing AML form ALL and in subclassifying AML, Assay of mutant p53 protein by immunophenotyping techniques. Detection of p53 gene mutation by PCR-SSCP and Sequencing techniques (ABI 310 genetic analyzer, Perkin Elmer).Assay of mutant p53 protein using FITC- conjugated monoclonal mouse anti-Human p53 protein clone DO-7 Code No.7054 Lot 050. Edition 15.06.00 *Results.* In this study we found that, the incidence of p53 mutations were higher in ALL patients (13.3%) than AML patients (7.6%) cases who showed p53 mutations at diagnosis were among cases who resisted chemotherapy (3cases out of cases), they had exon 5 and 6 mutations.P53 mutations showed higher incidence in relapsed AML and ALL patients (20% and 60% respectively), Exon 8 mutations were the most frequent type of mutation, affecting mainly relapsed AML and ALL. Followed by exon 6 and exon 7 mutations, that were restricted to relapsed ALL cases summery and conclusions P53 mutations were present infrequently in de nove acute. Leukemia higher incidence of p53 mutations were in aggressive and relapsed leukemia ALL was associated with higher incidence of p53 mutations than AML.The p53 mutations-bearing patients did not differ in their clinical and laboratory data from those without mutations Mutations of exon 8 were found in high frequency in relapsed acute leukemia (ALL and AML).mutations of exon 5 were found in acute leukemia patients with poor response to chemotherapy Mutations of exon 6 were found in patients with poor response to chemotherapy and in relapsed ALL Mutations of exon 7 were found in relapsed ALL Mutations of p53 usually affect both alleles with positive (LOH).

Stem cell transplantation - Experimental

0343

MATURATION OF NK CELL PHENOTYPE AFTER ALLOGENEIC HEMATOPOIETIC CELL TRANSPLANTATION

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Background. NK cells play a role in anti-infectious and anti-leukemic reactions after allogeneic hematopoietic cell transplantation (alloHCT), which is related to the expression of various stimulatory and inhibitory receptors. Although quantitative reconstitution of NK cells after alloHCT is fast, their phenotypic maturation is gradual and is expected to eventually resemble the donor-type pattern. *Aims.* To define factors influencing NK cell maturation as well as to analyze the impact of NK cell phenotypic reconstitution on outcome after alloHCT. *Methods.* Twenty-three patients with hematological malignancies treated with alloHCT from either HLA-identical sibling (n=8) or unrelated donor (n=15), were examined peripheral blood NK cell phenotype before transplantation (both donors and recipients) as well as on days +28, +56, +100 and +180 after alloHCT. In addition, killer immunoglobuline-like receptor (KIR) genotype of donors and recipients was studied before transplantation. For phenotyping we used antibodies specific for KIR2DL1/S1, KIR2DL2-3/S2, KIR3DL1, and NKG2A. Individual pattern was created for each patient and his donor based on frequencies of NK cells expressing respective receptors together with median fluorescence intensity (MFI). To quantify the differences between donor and recipient NK cell phenotype pattern at various time-points after alloHCT, we developed a new discrepancy index based on sum of differences defined as distances in coordinate system constructed by frequencies and MFI axes normalized by standard deviations. *Results.* Discrepancy index equaled 8.6 (±2.5) on day +28 and dropped to 8.2 (±2.7) (p=NS) on day +56, 7.0 (±3.0) (p=0.03, vs. baseline) on day +100, and 5.9 (±2.1) (p=0.0002, vs. baseline) on day +180. On days +28, +56, and +100 the index was higher for patients given transplant from unrelated donors compared to sibling alloHCT (p=0.03, p=0.05, and p=0.02, respectively). On day +100, values of the discrepancy index were increased in case of HLA-C incompatibility (p=0.005) as well as for patients who had experienced acute GVHD grade II-IV (p=0.02). In a multivariate analysis the effect was independent for both above factors. No other factor including the genotype of KIRs and KIR ligands, nor other patient-, donor-, and procedure-related variables was found to influence significantly NK cell phenotype pattern after alloHCT. The probability of the overall survival at 10 months was higher for patients with the discrepancy index <8.27 (median) vs. >8.27 on day +28 (100% vs. 63%, p=0.02). The difference resulted mainly from the incidence of fatal infections, which occurred after engraftment and were a cause of non-relapse mortality (0% vs. 28%, respectively, p=0.05). *Conclusions.* The pattern of NK cell phenotypic reconstitution after alloHCT generally tends to recapitulate the donor-type, but is negatively affected by the incidence of acute GVHD and HLA-C incompatibility. In turns, delayed maturation seems to be associated with impaired anti-infectious defense translating into decreased survival. The proposed discrepancy index appears feasible to quantify the differences between the donor and recipient NK cell phenotype pattern.

0344

CD 3/28 COSTIMULATION INCREASES CHRONIC GRAFT-VERSUS-HOST-INDUCING CAPACITY OF HUMAN T CELLS IN RAG2$^{-/-}$GAMMAC$^{-/-}$ MICE

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Background. Currently, transfer of huPBMC into immunodeficient mice is the only way to investigate human T cell function *in vivo*. We recently developed a model for reproducible engraftment of human T cells and induction of xenogenic GVHD by iv transfer of huPBMC into RAG2-/-γc-/- mice. *Aims and Methods.* Here we report our cumulative experience from experiments involving > 500 huPBMC-RAG2-/-γc-/- human-to-mouse chimeras and present new data and extended data to previously

published results. *Results.* Acute lethal X-GVHD generally occurs within 3 weeks after iv transfer of fresh huPBMC (15x106 CD3+ cells) into RAG2-/-γc-/- mice. X-GVHD occurred in 99% of 68 mice, including 87% acute lethal X-GVHD and 12% chronic X-GVHD. For 14 different donors, no significant difference between donors was observed with regard to development of acute X-GVHD. Acute X-GVHD could be effectively prevented by FK506 sc at day 0 resulting in 100% survival of mice, in contrast with FK506 administered at later time points. Treatment with prednisolone iv or OKT3 sc did not abrogate acute X-GVHD nor did IL-2 ip exacerbate acute X-GVHD. Further analysis showed the overall impact of *ex vivo* culture on development of X-GVHD for 216 mice. There was a significant linear correlation for fresh huPBMC in dose-dependent survival of 86 mice (r2 = 0.8, $p<0.008$) and for cultured huPBMC in 130 mice (r2= 0.8, $p<0.007$). In contrast to fresh huPBMC, only 44% of mice developed X-GVHD after injection of huPBMC (15x106 CD3+ cells) that were cultured and stimulated with OKT3, including 25% acute lethal X-GVHD and 19% chronic X-GVHD. Strikingly, this was different for CD3/28 costimulated huPBMC, of which 58% developed X-GVHD, including only 10% acute lethal X-GVHD and 48% chronic X-GVHD. These results suggest that CD3/28 costimulation of huPBMC stimulates the development of chronic X-GVHD. We speculate that a more efficient activation by CD28-ligation leads to an increase in *in vivo* survival and proliferation of human T cells that permits the development of chronic X-GVHD. *Conclusion.* The huPBMC-RAG2-/-γc-/- xenogeneic transplant model can be considered the most sensitive model to date for evaluation of human T cells *in vivo* and will be a valuable addition to current allogeneic murine T cell models. Future studies will involve further exploration of the influence of CD3/28 costimulation on development of chronic X-GVHD.

0345

CD4+CD25+ REGULATORY T CELLS ARE GENERATED AFTER BONE MARROW TRANSPLANTATION WITH REDUCED INTENSITY CONDITIONING REGIMENS IN AN ANTIGEN SPECIFIC FASHION

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CD4+CD25+ regulatory T cells are generated after bone marrow transplantation with reduced intensity conditioning regimens in an antigen specific fashion. *Background.* It has been shown that graft-versus-tumour (GvT) effect plays a prominent role in eradicating the underlying malignancy after allogeneic bone marrow transplantation (BMT) for leukaemia patients. The therapeutic GvT effect relies on the establishment of mixed haematopoietic chimerism which produces host-versus-graft (HvG) tolerance and allows donor lymphocyte to be infused and expanded in the recipient. Reduced intensity conditioning (RIC) regimens has been developed to assist the establishment of HvG tolerance in patients and animal models. *Aims.* To investigate the mechanisms of HvG tolerance after RIC-BMT. *Methods.* Female C57BL/6 mice were transplanted with male bone marrow cells after sublethal total body irradiation (400cGy). Under these conditions, male donor cell engraftment was achieved and HvG tolerance established within 4 weeks after BMT. FACS analysis was carried out to numerate CD4+CD25+ and CD4+FoxP3+ cells in recipient mice. Unfractionated splenocytes or CD4/CD25 depleted cells from chimeric mice were added to MLRs to test their suppressive ability *in vitro* on the proliferation of male-specific T cells in response to HY as measured by H-2b/HY-peptide tetramer staining. The immunosuppressive effect of unfractionated or CD4/CD25-depleted chimeric splenocytes was also tested *in vivo* using CFSE-labelled male/female cell mixture as targets. The effect of chimeric splenocytes was also assessed for their ability to favour engraftment of male BM cells in female recipient mice. Donor cell engraftment was detected using CD45 polymorphisms. *Results.* A significant increase in the percentage of CD4+CD25+ and CD4+FoxP3+ cells was observed in the peripheral blood of mice after 400cGy TBI. Splenocytes from chimeric mice were able to inhibit the proliferation of HY-specific CD8+ T cells as determined in the MLR experiments. Depletion of CD4+ or CD25+ cells from the chimeric splenocytes abolished their immuosuppressive ability. The adoptive transfer of chimeric splenocytes prevented *in vivo* killing of CFSE-labelled male donor cells in the female hosts, and enhanced donor cell engraftment. Also this ability was abolished by the depletion of CD4+ or CD25+ cells. When female BM was used to transplant to female recipient, although CD4+CD25+ still expanded, they failed to exhibit suppressive activity *in vitro* as well as *in vivo*. *Conclusion.* We conclude that CD4+CD25+ Tregs are generated as a consequence of RIC allografting. This expansion may play an important role in determining and maintaining HvG tolerance. Although an increment in the number of Tregs can be observed also following syngeneic transplantation, no immunosuppressive activity is exerted by the Tregs generated in this setting. Our results argue in favour of an antigen-driven mechanism for RIC-induced Tregs expansion.

0346

IMATINIB IMPAIRS PROLIFERATION AND FUNCTION OF CD8+ T LYMPHOCYTES S PECIFICALLY DIRECTED AGAINST THE LEUKEMIA-ASSOCIATED ANTIGEN RHAMM/CD168

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Background. The competitive Bcl-Abl tyrosine kinase inhibitor imatinib (STI571, Gleevec) is highly effective in the treatment of patients with chronic myelogenous leukemia (CML), and is increasingly used in patients with residual disease or relapse after allogeneic stem cell transplantation (allo-SCT) setting. *Aims.* Since the graft-versus-leukemia (GVL) effect after allo-SCT depends on T-lymphocytes and an impairment of anti-viral CD8+ T lymphocyte function has been described, we questioned whether imatinib also affects anti-leukemic CD8+ T lymphocytes. *Methods.* After eight days of mixed lymphocyte peptide culture (MLPC), we assessed CD8+ T cells from the peripheral blood of healthy volunteers and patients with CML by tetramer staining, multi-color flow cytometry and enzyme linked absorbent spot (ELISPOT) assays. *Results.* The release of interferon-γ and granzyme B by CD8+ T lymphocytes specific for the RHAMM3, a recently described T cell epitope peptide derived from the leukemia-antigen receptor for hyaluronic acid mediated motility (RHAMM/CD168) was inhibited by imatinib in a dose-dependent fashion. This inhibitory effect could not be ascribed to an increased rate of apoptosis. The inhibition of CD8+ T lymphocyte function was reversible after removal of imatinib from the MLPC after day four. Moreover, administration of imatinib to patients with CML decreased the functional activation of CD8+ T lymphocytes *in vivo* compared with the T cells of the same patients after cessation of imatinib. *Conclusion.* In the light of these findings, administration of high-dose imatinib might result in the reduction of efficacy of GVL or T cell based therapies.

0347

BORTEZOMIB INDUCES APOPTOSIS AND MODIFIES THE MATURATION PATTERN OF DENDRITIC CELLS: ROLE IN THE INDUCTION OF IMMUNOTOLERANCE AFTER ALLOGENEIC TRANSPLANTATION

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Background. The NF-kB family has emerged as a key transducer of inflammatory signals involved in dendritic cell (DC) maturation. Accordingly, stimulation of Toll-like receptors (TLR) by ligands such as lipopolysaccharide (LPS) or CpG-containing DNA induce DCs maturation through NF-kB activation. Thus, the proinflamatory milieu generated in the context of transplantation favours DCs maturation and increases their T cell priming abitity, so that DCs play a key role in the development of GVHD. Bortezomib is a potent, selective and reversible inhibitor of proteasome that blocks the nuclear translocation and transcriptional activity of NF-kB. *Aim.* In the current study we have analyzed the effect of bortezomib in both DCs' viability and maturation as well as the ability of bortezomib-treated DCs to generate a tolerogenic T-cell response. *Results.* Bortezomib showed a detrimental effect on DCs viability at 50nM and it was specially evident in cases cultured in the presence of TNFa and LPS while cell viability was not significantly affected in cultures performed without TNFa and LPS, indicating that bortezomib induced apoptosis of DCs in conditions which induce fully DCs activation. We next tested the effect of bortezomib on the expression of coestimulatory molecules and on the cytokine pattern of DCs. Interestingly, the addition of bortezomib decreased the expression of CD86 both in un-stimulated or fully activated (TNFa and LPS treated) DCs while the expression of CD80, CD40 and HLA-DR was also modified at 10nM of the drug. Concerning the cytokine pattern, the intracellular expression of IL-12 significantly decreased at a concentration of 10nM of the drug. In addition, we evaluated the effect of the DCs

cultured with or without bortezomib at 10 nM in the activation pattern and cytokine profile of T-cells after mixed lymphocyte cultures (MLRs) and we found that, among MLRs performed using un-stimulated DCs cultured without the drug, 20% (95% CI = 5.5-81) of T lymphocytes were activated as compared to 43% (95% CI = 11.5-88) among T cells co-cultured with DCs fully activated with TNFa and LPS (p=0.02); by contrast, the percentage of activated T cells were 21% (95% CI = 4.9-82) vs 31% (95% CI = 16-48) (p=0.34) when they were co-cultured with bortezomib-treated CDs non-activated vs fully activated with TNFa and LPS, respectively. This results indicated that bortezomib-treated DCs were unable to properly stimulate T cells even after exposure to a proinflamatory milieu. Moreover, T lymphocytes previously cultured with bortezomib-treated CDs were unable to become activated when they were further stimulated with fully activated untreated DCs from the same donor, indicating that T lymphocytes exposed to bortezomib-treated DCs become tolerant to the antigens presented by DCs. *Conclusion.* Bortezomib affects viability and modifies the maturation and cytokine pattern of DCs. The latter effect results in an impaired capability to induce allogeneic T cell stimulation and generates a tolerogeneic response of T cells cultured with bortezomib-treated DCs. These results suggest a potential role for the *in vivo* use of the drug prior to allogeneic transplantation through its effect on host DCs and/or for the *in vitro* generation of tolerogeneic DCs.

0348

STEM CELL FACTOR ENHANCES T-CELL RECOVERY AND THYMOPOIESIS FOLLOWING EXPERIMENTAL BMT

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Deficient thymopoiesis and retarded recovery of newly developed naïve CD4+ T-cells is one of the most important determinants of impaired immune competence following hematopoietic stem cell transplantation (HSCT). Recently, we showed that Fms-like tyrosine kinase-ligand 3 (FL) accelerates T-cell recovery following experimental bone marrow transplantation (BMT) via expansion of bone marrow (BM) lymphoid progenitors prior to recovery of thymopoiesis. Several studies have suggested an important role for stem cell factor (SCF)-c-kit interactions in T-cell development, but is unclear at which level SCF primarily affects T-cell development. Here we evaluated whether SCF would affect T-cell recovery, thymopoiesis and BM lymphoid progenitor cell numbers following experimental BMT. Three Gy irradiated Rag-1-/- mice (C57Bl/6 Ly5.2 background) were used as recipients of T-cell depleted congeneic bone marrow cells (4×10^4 C57Bl/6 Ly5.1 origin). Mice were treated with PBS, SCF (100 µg/kg s.c.), FL (800 µg/kg) or SCF combined with FL 3 times weekly for 4 weeks following BMT (n=6/group). Peripheral blood (PB), splenic and thymic lymphocyte subsets and BM lymphoid progenitors (LSKflt3-, LSKflt3+, common lymphoid progenitors (CLP)) were quantified using FACS-analysis at day 28 post-BMT. SCF- or FL-treated mice showed higher numbers of both PB and splenic T-cells as compared to PBS-treated control mice (PBS vs. SCF (mean absolute splenic T-cell numbers $\times 10^6$ /spleen±SEM): 0.3±0.1 vs. 5.4±2.8; p=0.02). No additive or synergistic effect was observed in mice treated with both SCF and FL. In contrast to FL, SCF did not increase peripheral B-, NK and dendritic cell numbers. SCF- or FL-treated mice showed an increase in thymic cellularity (PBS vs. SCF (mean absolute cell numbers $\times 10^6$ /thymus ±SEM): 6.2±0.2 vs. 11.4±4.1; p=0.46), numbers of donor-derived thymocytes (0.13± 0.08 x 106 vs. 7.2± 4.5; p=0.06) and numbers of all thymocyte subsets, including DN (0.5 × 104±vs. 10.4 ±; p=0.07), DP (10.7± 7.4 vs. 470± 356; p=0.1), CD4SP (1.3 ±0.7 vs. 16 ±8; p=0.08) and CD8SP (0.3 ±0.1 vs. 69±32; p=0.04). In addition a trend towards increased percentages and absolute numbers of BM LSKflt3+ was observed in SCF-treated mice (PBS vs. SCF (mean absolute BM LSKflt3+ cell numbers ×10*3/femur ±SEM): 8±4 vs. 35±24; p=0.12). These data show that SCF or FL may enhance T-cell recovery and thymopoiesis. However, in contrast to FL, SCF did not increase other peripheral lymphoid cell lineages, but selectively promoted T-cell recovery. Collectively these data suggest, that SCF may enhance T-cell recovery by improvement of thymopoiesis and possibly also by expansion of lymphoid progenitors after BMT. These results may provide a rationale for clinical application in recipients of HSCT with a retarded T-cell recovery mainly due to transplantation of limited numbers of progenitor cells such as may occur in cord blood transplantation.

0349

IS BODY-WEIGHT-BASED CALCULATION OF IV BUSULFAN FIXED DOSE THE APPROPRIATE DOSAGE OF BUSULFAN IN CHILDREN UNDERGOING HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Background. High-dose oral busulfan (Bu) is often included in conditioning/preparative regimens prior to autologous (auto-) or allogeneic (allo-) transplantation (T). Studies have reported that pharmacokinetics (PKs) of Bu in HSCT are age-dependent with underexposition of children received the usual dosage 16 mg/kg over 4 days. Bu clearance (Cl) is highly variable in children and increased in the youngest. Thus age-based dosing and therapeutic drug monitoring (TDM) with dose adjustment are needed to target an area-under the curve-plasma-concentrations (AUC) equivalent to adults. An IVBu form was developed. In a first study (US trial), 24 children received an IVBu age-based dosing with TDM equivalent to the oral (1.0 mg/kg ≤ 4y and 0.8 mg/kg > 4y) (Wall D., ASH 2000, #2066). A retrospective analysis suggested that the dose of Bu should rather be calculated on the basis of the body-weight (BW) (Nguyen L et al BMT 2004). To validate the new dose-regimen a prospective study was conducted. *Aims.* To prospectively validate a new body-weight-based fixed dose of IVBu in children in its ability to target an AUC within a predefined therapeutic window for more than 75% of patients without any therapeutic drug monitoring. *Methods.* PKs of IVBu administered at the defined dosage were studied in children who received either IVBu/Melphalan or IVBu/Cyclophosphamide prior to auto- or allo-T, respectively. IV Bu (16 doses) were administered over 2 h at 1.0 mg/kg, 1.2 mg/kg, 1.1 mg/kg, 0.95 mg/kg, and 0.8 mg/kg for patients (pts) with <9 kg, 9 to <16 kg, 16-23 kg, >23-34 kg, and >34 kg strata of body weight, respectively. PK was performed at doses 1, 9 and 13 but no dose adjustment was allowed. Bayesian Bu AUCs were calculated. *Results.* Preliminary results are available in 55 pts, median age 6y [0.3-17.2], including 20 pts < 4 y. A significantly better AUC targeting (900-1350 µM.min) was achieved with the new fixed dose as compared to the usual age-based dosing (76% vs. 54%, p= .001). Moreover, there is no longer a significant difference in systemic exposure (mean±s.d.: 1248±205 µM.min) between children treated with this new dosage and adults given 12.8 mg/kg of IVBu, although significant differences (p=0.001) on Bu Cl were observed among weight groups. AUC inter-pt variability was 2.5 fold reduced (CV ≤ 20%). Low-intra pt variability (CV ≤ 10%) enabled reproducible exposure without the use of TDM in children. All AUCs were > 847 µM.min, and 84% < 1500 µM.min which may explain the high rate of engraftment (no graft rejection), and the low incidence of veino-occlusive disease of the liver (4/27 auto-T, 2/28 allo-T) that previously correlated with low and high Bu exposure, respectively. *Summary/Conclusions.* We conclude that BW-based IVBu dose regimen enabled reproducible AUCs (without TDM) throughout the treatment period as compared to age-based dosing and oral Bu with PK monitoring. Based on this prospective study, the defined IVBu doses according to BW are recommended in children.

0350

TRANSPLANTATION OF HUMAN PERIPHERAL BLOOD CD34-POSITIVE CELLS IN COMBINATION WITH *EX VIVO* EXPANDED MEGAKARYOCYTES IN NOD/SCID MICE

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The period of severe thrombocytopenia after high dose chemotherapy may be shortened by increasing the number of megakaryocytes (MKs, CD41+ cells) in the stem cell transplant. The addition of *ex vivo* expanded MKs to the graft might suit this purpose. Our aim is to study the fate, engraftment ability and platelet production capacity of MKs expanded from mobilized peripheral blood (MPB) stem cells in a murine xenotransplantation model for human hematopoiesis, the NOD/SCID mouse In pilot experiments, we established that the detection threshold

of human platelets in mouse blood is 1×10^4/mL and that the injection of 0.3 μg (human) Tpo right after transplantation of unmanipulated MPB stem cells does not affect the numbers of human blood platelets or the percentage of human hematopoietic cells in the mouse bone marrow (BM). Next, MPB CD34+ cells were cultured for 7 days in the presence of Tpo (100 ng/mL) and IL-1β (10 ng/mL). An expansion of approximately 6-fold was observed after 7 days of culture. Over 50% of the expanded cells expressed CD41, but limited numbers of CD34 expressing cells were detected. After sublethal irradiation, NOD/SCID mice were transplanted with unmanipulated CD34+ cells (group A), unmanipulated cells combined with *ex vivo* generated MKs (group B, C, D), or *ex vivo* generated MKs only (group E)(see Table 1 for the dosing scheme). As control, the mice of group F did not receive any cells after irradiation. Blood was collected at day 3, 7, 10, 14, 21 and 28 after transplantation. Already after three days human platelets could be detected in the blood of the mice that received the highest number of cultured cells (group C and E). After 7 days, human platelets were detected in the blood of the mice from all groups, except the mice of group A, which received only uncultured cells. In the mice of the groups A, B, C and D platelets numbers increased till day 14 (to an average of 6.9×10^6/mLblood) with a small decrease towards day 21 (5.9×10^6/mL) and day 28 (4.5×10^6/mL). The mice of group E reached a maximum of 3.4×10^5 human platelets per ml blood at day 10 and numbers declined from thereon. At day 21 human platelets in the mice of group E were hardly detectable. The experiment will be terminated at day 35 and chimerism will be determined in the blood, BM and spleen. In summary, expanded MKs can significantly contribute to thrombocytopoiesis during the first days after transplantation. This indicates that the period of thrombocytopenia after intensive chemotherapy can be overcome by the co-transplantation of *ex vivo* expanded human MPB MKs. Since previously published clinical trials showed only a small effect of co-transplanted MKs it may be interesting to extend our protocol to a clinical setting.

Table 1.

Group	Cells	Cells transplanted		Mice
A	MPB CD34+	4.5×10^6 NC/mouse	100%	4
B	MPB CD34+ + expanded CD34+	4.05×10^6 NC + 0.45×10^6 NC input culture/mouse	90%+10%	4
C	MPB CD34+ + expanded CD34+	4.5×10^6 NC + 4.5×10^6 NC input culture/mouse	100% + 100%	4
D	MPB CD34+ + expanded CD34+	2.25×10^6 NC + 2.25×10^6 NC input culture/mouse	50% + 50%	4

0351

THE T CELL RECEPTOR REPERTOIRE USAGE DIFFERS BETWEEN CD4+CD25+ REGULATORY T CELLS AND THEIR CD4+CD25- COUNTERPART AFTER ALLOGENEIC STEM CELL TRANSPLANTATION

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Background. After allogeneic haematopoietic stem cell transplantation (SCT) the overall T cell receptor (TCR) repertoire is characterized by a lower diversity and a markedly skewed pattern. Its normalization may start at about 6 months after transplant but most patients continue to show an abnormal profile until 2 or 3 years. Naturally occurring CD25+ regulatory T (Treg) cells also develop in the thymus and play a crucial role in the maintenance of peripheral tolerance. Although it is known that the administration of Treg cells has a protective effect in murine models of acute graft-versus-host disease, their role after SCT in humans has not been fully elucidated. *Aims.* We assessed whether naturally occurring Treg lymphocytes exhibit an impairment in their TCR repertoire after transplantation, similar to what observed in conventional T cells. We analyzed the TCR Vβ CDR3 repertoire of CD4+CD25+ Treg cells after allogeneic SCT, focusing on its overall complexity and on the degree of similarity to CD4+CD25- conventional T (Tconv) cells. *Methods.* We analyzed 10 patients who had received SCT for chronic myeloid leukemia. After CD4+CD25+ and CD4+CD25- cell isolation, RNA extraction and reverse transcriptase PCR, CDR3 region fragment analysis was performed through capillary electrophoresis. Conventional spectratyping evaluation was carried out by calculating an overall complexity score and by determining the percentage of skewed and oligoclonal Vβ profiles. Moreover, we developed a new analysis method to quantify the proportion of Vβ subfamilies with similar profile between the Treg subset and its Tconv counterpart. *Results.* Although we observed a significantly higher percentage of skewed and oligoclonal Vβ subfamilies in both cell subpopulations in patients less than 3 years after SCT, the conventional analysis systems showed essentially similar TCR patterns between Treg and Tconv cells. We then compared the spectratyping profiles of the 2 cell subsets within each Vβ subfamily in each subject. As a tool we developed a new *similarity score*, expressing the proportion of Vβ subfamilies with similar profile between Treg and Tconv subsets. We detected a positive correlation between similarity score and time after SCT (Pearson correlation coefficient = 0.65). A higher score was observed in patients more than 3 years after allografting (mean 0.90 vs. 0.61, p= 0.01). Noticeably, in patients less than 3 years after SCT the differences were very often ascribable to the detection in the same Vβ subfamily of an oligoclonal profile in the Tconv but not in the Treg subpopulation. This specific pattern was almost exclusively confined to this group of patients (mean 52% vs. 5%, p= 0.002). *Conclusions.* Our data show that the repertoires of Treg and Tconv cells exhibit significant differences early after SCT, while they tend to become identical with full reconstitution. These differences are mainly ascribable to Vβ subfamilies expressing an oligoclonal profile in the Tconv but not in the Treg subset and could either reflect a discrepancy in the *in vivo* reactivity against common antigenic stimulations or be the result of a different post-transplant ontogeny.

0352

THE GRAFT VERSUS HOST DISEASE AND SURVIVAL MIGHT BE DIFFERENT ACCORDING TO ADMINISTRATION ROUTE AND DOSE OF MESENCHYMAL STEM CELL LINE IN MHC MISMATCHED MURINE HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Background. The stable engraftment and graft versus host disease (GVHD) should be overcome in allogeneic hematopoietic stem cell transplantation (HSCT). Mesenchymal stem cells (MSC) contributed to sustain early engraftment and lesser GVHD. In conventional HSCT, a lot of cells were sequestrated into liver or lung during intravenous infusion. *Aims.* We evaluated whether survival and GVHD in HSCT would be different according to administration route, and dose of MSC. *Methods.* We retrieved MSC through 5 consecutive subculture of C3H/10T1/2. All lethally irradiated 6 weeks-old female Balb/c mice received 1×10^7 bone marrow cells and 5×10^6 spleen cells of female C3H/He mice according to route and dose. The study groups were divided into the intravenous (IV) and intra-marrow injection (IBM) according to route, and also dose of MSC. In co-administration of MSC, mice were designed in HSCT with 1×10^5 MSC and 1×10^6 MSC, and some mice received with addition of MSC on post-HSCT 48 hours. All mice were observed daily for survival and GVHD clinical status. *Results.* All mice without MSC died with no different GVHD pattern in post-HSCT 8 day in spite of route. In HSCT with MSC, there were no difference of survival rate and GVHD score in mice co-transplantation with 1×10^5 MSC, and also with addition of MSC on post-HSCT 2 day in both IV and IBM group. However, mice received with 1×10^6 MSC were significantly better survival and lower GVHD score than others in both groups, although mice in IV group were longer survival than in IBM group. *Conclusions.* Our data suggested that the administration route of cells would not affect survival and GVHD pattern, and co-transplantation with high dose of MSC might prevent lethal GVHD in MHC mismatched allogeneic murine HSCT. We concluded that HSCT with IV infusion of high dose of MSC might prevent lethal GVHD and have survival benefit.

0353

TWO ISOFORMS OF HUMAN FOXP3 POSSESS SIMILAR CAPACITIES TO INDUCE DIFFERENTIATION OF REGULATORY T CELLS FROM CD4+CD25- T CELLS

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Background. Naturally occurring CD4+CD25+ regulatory T cells (Tregs) are considered to play important roles in the clinical outcome of stem cell transplantation (SCT). The forkhead/winged helix transcription factor, Foxp3 is the key factor for the differentiation of Tregs. While in rodents the Foxp3 gene is expressed as a single transcript, in humans it

is usually expressed as two transcripts. One of these transcripts is the full-length Foxp3 (Foxp3FL). The other is an alternative RNA-splicing product, lacking the exon2 (Foxp3'E2). Currently it is not known whether these human foxp3 isoforms are expressed independently from each other and whether they are both involved in differentiation of Tregs. *Aims.* We investigated the cellular distribution of human foxp3 isoforms and their role in the differentiation of Tregs. *Methods.* The cellular distribution of foxp3 isoforms was analysed using quantitative PCR (QPCR) primer sets that amplify either the Foxp3FL or the Foxp3'E2 gene. The role Foxp3 isoforms in Treg-differentiation was studied by retroviral transduction of foxp3FL and Foxp3'E2 genes separately into highly purified CD4+CD25- Foxp3- cells. Foxp3 transduced T cells were cultured briefly and purified to >95% purity before phenotypical and functional characterization. *Results.* In PBMC of healthy individuals, both Foxp3 isoforms were preferentially expressed in CD4+CD25high cells. However, there was no quantitative relation between the gene expression levels of these isoforms. In Treg clones, generated by limiting dilution of CD4+CD25hi cells Foxp3 isoforms were expressed simultaneously; but there was no quantitative correlation between their expression levels. Phenotypic and functional analyses of foxp3 transduced T cells revealed that T cells transduced either with Foxp3FL or with Foxp3'E2 genes expressed high levels of CD25, CTLA-4 and GITR; were anergic to stimulation via CD3 and suppressed the CD3 induced proliferation of autologous and allogeneic CD4+CD25- cells in a dose dependent manner. *Summary and Conclusions.* Our results reveal that the two isoforms of human FOXP3 possess similar capacities to induce differentiation of Tregs from CD4+CD25- T Cells. Since they can be quantitatively expressed independently from each other, studies aiming at correlating Treg cell numbers with the clinical outcome of SCT will benefit from quantitative determination of both foxp3 isoforms.

0354

BODY-WEIGHT-BASED IV BUSULFAN FIXED DOSING AS PART OF BUMEL REGIMEN BEFORE AUTOLOGOUS TRANSPLANTATION IN CHILDREN WITH HIGH RISK SOLID TUMORS: REDUCED TOXICITIES IN THE FRENCH PROSPECTIVE MULTICENTRE STUDY

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Introduction. Oral busulfan (Bu) and melphalan (Mel) has been extensively used as a high-dose chemotherapy regimen followed by hematopoietic stem cell transplantation (HSCT) in pediatric patients (pts) with high-risk solid tumors. Bu has a narrow therapeutic window. With the availability of IV Bu, a new dosing strategy based on body weight (BW) has been defined allowing to target area under the curve (AUC) without any therapeutic drug monitoring (TDM) (Nguyen L. *et al.* BMT 2004). We assessed prospectively the new IVBu body-weight-based fixed dosing. The Pharmacokinetics results are reported separately (G. Vassal *et al.*), we report here the clinical outcomes of autologous pts. *Aims.* To investigate the safety of this new IVBu dosing strategy, to assess hematopoietic recovery, and to evaluate the consequences of IVBu dosage upon children clinical outcome. *Patients and Methods.* Twenty-seven children (14 male/13 female) received IV Bu over 2 h at a dose of 1.0 mg/kg, 1.2 mg/kg, 1.1 mg/kg, 0.95 mg/kg, and 0.8 mg/kg for pts with <9 kg, 9-< 16 kg, 16-23 kg, >23-34 kg, and > 34 kg strata of weight, respectively. Mel 140 mg/m^2 was then administered followed by HSCT. Clonazepam was given as seizures prophylaxis. Indications for HSCT were: high risk neuroblastoma [NB; n = 24: 9 CR1/ CR2, 10 VGPR, 5 PR1/PR2), Ewing's Sarcoma [EW, n = 3; 1 CR1, 2 PR1)]. Median age (range) was 4.0 y (0.7-14.9). Regimen-related toxicity (RRT) was graded according to NCI-CTC 2.0. Kaplan-Meier EFS and OS were evaluated. *Results.* No adverse effect was observed during IV Bu administration. Pts received 5.9 ×10^6/kg CD34+ (range 3-34) with post transplant G-CSF in 24/27. Median neutrophils (≥ 0.5×10^9/L) and platelets (≥ 50.0×10^9/L) recovery occurred at Day 11 (range 10-15) and 34 days (range 12-133), respectively. Digestive toxicity (mucositis) was the main RRT, grade I-II and grade III occurred in 24 and 14 pts, respectively. Four pts (15%, 95% CI: 4.2-33.7%) had moderate reversible veino-occlusive disease (VOD). There was no regimen-related death. 13/27 pts had disease relapse/ progression after a median time of 8.6 months (2.6'29.8), and 9/27 pts died. With a median follow-up of 31.6 months (range 20.1-41.2) estimated EFS and OS rates were as follows: 42%±26% and 64%±22%, for high-risk NB, respectively; and 67% ±27% for both probabilities for high-risk EW sarcoma. *Summary/Conclusions.* These results suggest that the IV administration of Bu with a BW dosing has no impact on efficacy of the BuMel regimen and decrease its toxicity, especially VOD.

0355

TELOMERE LENGTH OF HAEMATOPOIETIC CELLS FOLLOWING AUTOLOGOUS OR ALLOGENEIC TRANSPLANTATION REFLECTS THAT OF GRAFTED STEM CELLS

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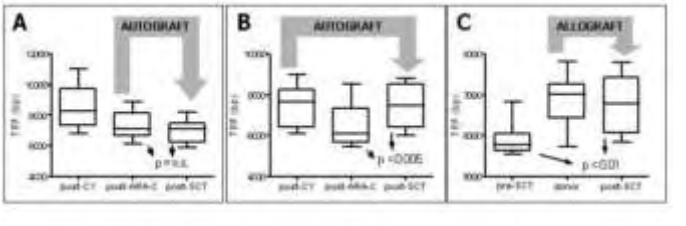
Background. In human somatic cells, telomere length decreases at each mitotic division and progressively shortens with age. Due to this peculiarity, telomere restriction fragment (TRF) length has been used as a marker of cell aging. The systematic analysis of TRF has suggested that haematopoietic stem cells (HSCs) may undergo early cell aging if exposed to non-physiological proliferative stress, as it occurs during haematopoietic reconstitution following high dose (hd) chemotherapy and stem cell transplantation (SCT). Information is still lacking about the influence of telomere length of grafted cells on the degree of telomere erosion following SCT. AIM OF THE STUDY. To correlate TRF length in grafted peripheral blood stem cells (PBSC) and in haematopoietic cells taken at marrow reconstitution following SCT. PATIENTS AND *Methods.* TRF length was monitored in a series of lymphoma patients undergoing an intensive high-dose sequential (HDS) program, including two consecutive mobilization procedures, with hd-cyclophosphamide (CY) and hd-Ara-C, and then PBSC autograft. In a previous report (Ricca I *et al.,* Leukemia 2005), we showed that TRF length is markedly shortened in PBSC collected at the second mobilization course compared to those collected at the previous one. Thus, TRF was assessed in 10 patients autografted with post-CY PBSC and 13 receiving post-Ara-C PBSC; in addition, TRF was assessed in 8 patients receiving allogeneic SCT. TRF was assessed both on grafted material and on bone marrow (BM) samples taken at a median time of 24 months after SCT. All patients were in complete remission of their underlying disease at the time of analysis. *Results.* As shown in the Figure (panels A and B), TRF length was markedly shortened in post-Ara-C PBSC compared to post-CY, with median TRF length of 8100 bp (range 6105-11024) and 7046 bp (range 5561-8906), following hd-CY and hd-Ara-C, respectively (p<0.0001). At post-SCT follow-up, median TRF length in patients autografted with post-Ara-C PBSC (panel A) was 7092 bp (range 5851-8227), thus analogous to pre-transplant value; by contrast, patients autografted with post-CY PBSC (panel B) had a significantly longer TRF length (7474 bp, range 6707-8808) compared to pre-transplant value (p<0.005). Panel C shows TRF values in the eight allografted patients: again, post-SCT TRF length was similar to the graft (p=NS) and markedly longer if compared to patient pre-SCT value (p<0.01). *Conclusions.* In the SCT setting, post-graft TRF length strictly correlates to TRF of grafted cells.



0356

PERMANENT TELOMERE LOSS IN MYELOID CELLS FROM LONG-TERM SURVIVORS OF LYMPHOMA PATIENTS TREATED WITH HIGH-DOSE CHEMOTHERAPY AND AUTOGRAFT

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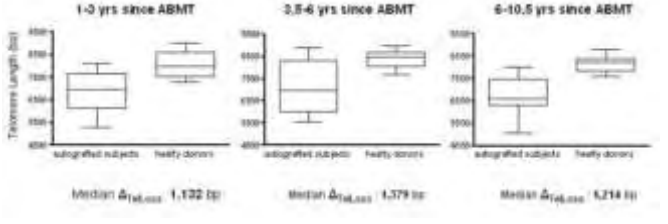
Background. Telomere length (TL) decreases at each cell division and therefore it is a good marker of cell replication history. Accelerated cell proliferation is required during post-autograft hematopoietic recovery

resulting in abnormal telomere loss at least in the early period after autologous bone marrow transplantation. *Aims.* to evaluate both TL and bone marrow (BM) hematopoietic progenitor cell reservoir in long-term survivors lymphoma patients treated with high-dose chemotherapy and peripheral blood stem cell (PBSC) transplantation. PATIENTS AND *Methods.* TL was assessed on Peripheral Blood (PB) and BM samples obtained from 41 subjects (17 female and 24 male) in continuous complete remission from a high-risk lymphoma, at 1 to 10.5 yrs. (median 5.8 yrs.) since autograft. All subjects were autografted with large amount of PBSC (median CD34+ve cells/kg: $5{,}3\times10^6$). Their median age at the time of transplant was 44 yrs. (range 18-65). TL was determined by Southern Blot analysis on mononuclear cells (MNC) from PB and BM samples and on PB granulocytes (GN). MNC were separated through a ficoll density gradient, while GN were obtained from PB using a double step separation: RBC sedimentation in the presence of 33% Emagel and then ficoll separation to obtain granulocytes. BM progenitors were investigated by the *in vitro* culture assays, and both committed (CFU-GEMM, CFU-GM, BFU-E) and immature (LTC-IC) progenitors were evaluated. *Results.* TL of separated PB granulocytes was significantly shorter in autografted subjects compared to age-matched healthy subjects. A similar reduction was observed on BM cells, while no significant differences were observed on whole PB leukocytes. TL loss appeared to be stable, since the median difference in granulocyte TL between autografted subjects and age-matched controls (TelLoss) was virtually the same in subjects at different time-periods since autograft (see Figure). Both immature and committed BM progenitors were found to be markedly reduced compared to normal controls (data not shown). Again, no correlation between degree of progenitor reduction and time-interval since autograft was observed. *Conclusions.* i. telomere length is reduced in myeloid cells from subjects surviving up to 10 years following autograft; ii. TL reduction is long-standing suggesting that telomere-elongating enzymes are unable to reconstitute a normal telomere length even after a prolonged period following autograft; iii. a marked reduction of BM immature and committed progenitors is also maintained at long-term in spite of the large amounts of transplanted CD34+ve cells. Thus high-dose chemotherapy and PBSC autograft may result in myelopoietic cell abnormalities that appear to be irreversible. This observation is of both biological and clinical relevance.



0357

HIGH DOSE CHEMOTHERAPY+AUTOLOGOUS STEM CELL TRANSPLANTATION IN MULTIPLE SCLEROSIS PATIENTS: TREATMENT OUTCOMES AT LONG-TERM FOLLOW-UP

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Background. During the last several years HDCT+ASCT is more often used as a therapeutic option for MS patients. The major treatment outcomes for MS patients are a period free of disease progression and improvement of a patient's quality of life (QoL). *Aims.* We aimed to study treatment outcomes in MS patients at long-term follow-up after HDCT+ASCT. *Methods.* Twenty-five patients with MS (secondary progressive - 13 patients, primary progressive - 8, progressive relapsing - 1, relapsing remitting - 3) were included in this study (mean age - 33.0, SD - 7.4; male/female - 8/17). Median EDSS at base-line was 6.0 (range 2.0 - 8.0). The median follow-up duration was 18 months (range 6-72 months). All of the patients had previously undergone conventional treatment. Neurological and QoL evaluation was provided at baseline, at discharge, 3, 6, 9, 12 months, and then every 6 months after HDCT+ASCT. MRI was conducted at baseline, at 6, 12 months, and at the end of follow-up. FACT-BMT and FAMS were used for QoL evaluation. QoL response was evaluated using Integral QoL index. *Results.* Twenty patients with the follow-up longer than 1 year were included in the analysis. 19 patients (95%) experienced a clinical stabilization or improvement. Three patients showed significant improvement in EDSS (by more than 1.0 point), 4 patients improved by 1.0 point, and 4 patients - by 0.5 points on EDSS. Eight cases remained stable. All of the patients with clinical stabilization and improvement exhibited negative MRI scans. One patient deteriorated to a worse score after 18 months of stabilization and died of acute leukemia 3 years post transplant. Two patients after 12 months of improvement progressed to a worse EDSS score.

0358

DAY 15 NATURAL KILLER (NK) CELL RECOVERY PREDICTS PROGRESSSION-FREE SURVIVIAL AFTER AUTOLOGOUS STEM CELL TRANSPLANTATION IN NON-HODGKIN'S LYMPHOMA

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Background. The peripheral blood absolute lymphocyte count (ALC) on day 15 after autologous stem cell transplantation (ASCT) has been shown to be an independent predictor for overall survival (OS) for many malignancies including acute myelogenous leukemia (AML), breast cancer, multiple myeloma (MM), primary systemic amyloidosis, and Hodgkin's and non-Hodgkin's lymphoma (NHL). However, due to the retrospective nature of previous studies, the peripheral blood lymphocyte subpopulations that predict survival are unknown. *Aims.* To prospectively correlate peripheral blood lymphocyte subpopulations on day 15 after ASCT and progression free survival in NHL. *Methods.* Peripheral blood lymphocytes collected from patients before and on day 15 after ASCT were analyzed by four color flow cytometry for CD3, CD4, CD8, CD16, CD 19, and CD56. Patients were then dichotomized into two groups: patients achieving normal numbers of lymphocyte subset (i.e. CD4, CD8, CD19, CD16/56) count versus those who did not. Progression free survival was then analyzed by the Kaplan-Meier method. *Results.* In our cohort of 14 patients, 9 were male and 5 were female. Nine patients were diagnosed with diffuse large cell B cell lymphoma, two with mantle cell, two with follicular, and one with peripheral T cell lymphoma. On presentation, one patient had stage I, four patients had stage II, four had stage III, and five patients had stage IV disease. The median age at ASCT was 53 years (range: 26-70). The preliminary data from this ongoing prospective study of 14 patients shows that on day 15 after ASCT, 4/14 (29%) of patients achieved a normal CD3 count (median 321, range: 69-2069 cells/μL) 3/14 (21%) achieved a normal CD4 count (median 206, range: 31-1091 cells/μL), 6/14 (43%) achieved a normal CD 8 count (median 88.5, range: 13-813 cells/μL) 1/14 (7%) achieved a normal CD19 count (median 2, range:0-227 cells/μL), and 8/14 (57%) achieved a normal CD16/56 count (median 85.5, range: 10-744 cells/μL). The median follow-up was 12 months (range: 3-45 months). On univariate analysis, patients achieving an absolute NK cell count of ≥ 80 cells/μ on day 15 had significantly improved progression free survival compared to those who did not (not reached vs 3 months, $p<0.006$, respectively). Similar analysis evaluating the absolute CD3, CD4, CD8, and CD 19 count was not significant ($p=0.1$, $p=0.258$, $p= 0.06$, and $p=0.55$, respectively). *Conclusion.* To our knowledge, this is the first report detailing the critical role of NK cell immune reconstitution after ASCT in progression free survival.

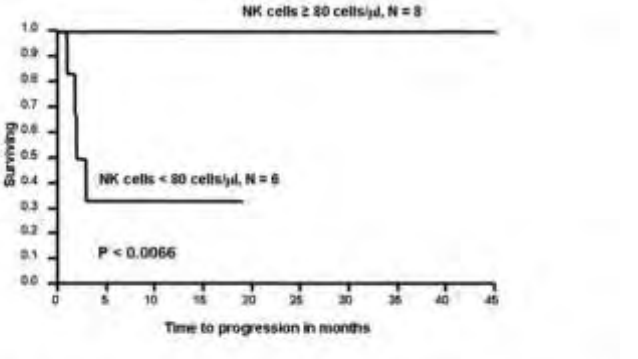

Figure 1: Kaplan-Meier curve of time to progression in patients achieving an NK cell count of ≥80 cells/μl compared to patients with an NK cell count <80 cells/μl at day 15 after ASCT

0359

AM3 THERAPY PREVENTS MUCOSITIS IN PATIENTS UNDERGOING HEMATOPOIETIC STEM CELLS TRANSPLANTATION

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Introduction. AM3(Inmunoferon®), is a glycoconjugate of natural origin with immunomodulatory properties indicated in secondary immnunodeficiencies as well as coadjuvant treatment in neoplastic diseases with cellular immunity deficiency. *Aim.*To evaluate the influence of AM3 in the biological recovery of immune system in patients undergoing hematopoietic stem cells transplantation (HSCT)(autologous/allogenic). As well as to register the clinical incidences as undercurrent infections and mucositis from day of infusion hematopoietic cells to 90 days postransplantion in patients with and without AM3. Patients and *Methods.* Group A (cases): Inclusion of 19 consecutive patients undergoing HSCT. April 2004 - June 2005. Inclusion criteria: age > 18 years, first HSCT and signed consent form. AM3 dose: 20mg/8h orally since infusion day to 90 days postransplantation. Group B (controls): 19 patients consecutively undergoing HSCT from February 2003- March 2004. Biological evaluation of the immune system recovery: BCC, immunoproteins quantification: IgG, IgA, IgM, C3, C4, lymphoid populations distribution and activity of monocyte biomarkers (chitotriosidase, CCL18/PARC) on days '7, 0,+7,+14,+21,+28,+56,+90. Clinical evaluation and comparing cases/controls of incidence of mucositis severity evaluated according to WHO classification and microbiological documented infectious diseases. *Results.* The addition of AM3 as a coadjutant therapy in patients undergoing HSCT reduces significantly the percentage of oral mucositis (63.1% vs 89.5%) (p=0.025) being those mucositis of less complexity in the 2 first weeks postransplantation. The number of infectious diseases was lower in patients under AM3 therapy (31,5% vs 42%) (p=0.254) (Detailed in table). The recovery of the immune system evaluated by blood cells count and immunobiomarkers showed a initial recuperation at day +14 in autologous HSCT and at day 21 for allogenic HSCT. The tolerance was satisfactory and only two patients needed discontinue therapy because of digestive intolerance. Wide studies must be performed in order to evaluate the benefit of this coadjutant therapy. This work has been partially sponsored by a grant from FEHHA.

Table 1.

Mean age (range)	*Group A 44 (18-67)*	*Group B 48 (18-68)*
Females	6 (31%)	7 (36,8%)
Allogeneic HSCT (M/F)	4 (21%) (3M/1F)	6 (31,5%) (5M/1F)
Autologous HSCT (M/F)	15 (78,9%) (10H/5M)	13 (68,4%) (7M/6F)
Diagnosis	Acute leukemia: 7 (37%), MM 7 (37%), MDS 2 (10%), Hodgkin disease 2nd remission 2(11%) lymphoblastic lymphoma 1 (5%)	Acute leukemia: 4 (21%), MM 9(47%), Hodgkin, disease 2nd remission 2(11%), NHL 4 (21%)
Mucositis	12 (63%): grade I: 1 patient (9%) grade II: 4(33%); grade III: 4 (33%); grade IV: 3 (15%)	17 (89,5%): grade II: 1 patient (6%); grade III: 6 (35%) grade IV 9 (53%)
Infectious diseases	6 (31,5%): S. Epidemidis 4 (21%) St. homonis 1 (5%), CMV 2 (10,5%), C. Albicans 1(5%)	8 (42%) St coagulase negative 7 (36,8%), Str viridans 1(5.2%), S. epidermidis 1 (5.2%), Ochrobactrum anthropi 1 (5.2%) P. aeruginosa 1(5.2%), C. difficile 1 (5.2%), ADN Herpes virus 6: 2 (10.5%), CMV 3 (15.7%), C. albicans 2 (10,5%), C Krusei 1 (5,2%), P. carinii 1 (5,2%)

0360

INFLUENCE OF THE ADMINISTRATION ORDER OF BUSULFAN AND CYCLOPHOSPHAMIDE ON THE ENGRAFTMENT AND CHIMERISM IN SYNGENEIC STEM CELL TRANSPLANTATION MOUSE MODEL

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Background. Although stem cell transplantation (SCT) is considered to be a curative therapy for malignant and non-malignant diseases, still GVHD is a limiting factor. Several studies have shown that cells that are involving in initiation and promoting of GVHD are dendritic and T regulatory cells. It has been shown that GVHD is mainly due to of the imbalance (activation or suppression) between these two groups of cells. This disparity somehow relates to the intensity of different chemo-radiotherapy conditioning on the recipients and cytokine storm preceding the SCT. *Aims.* Our aim was to study the influence of administration order of busulphan (Bu) and cyclophosphamide (Cy) on the chimerism and engraftment of the dendritic and regulatory T-cells. *Methods.* Sixty female Balb/c mice were divided in two groups. Group I (Cy-Bu) received Cy (100 mg/kg/day, two days) followed by liposomal Bu (15 mg/kg/day, four days) and group II (Bu-Cy) received the same dose of the drugs but in reversed order. Forty two of recipients were transplanted using Sca-1 from Balb/c males. The chimerism and engraftment of dendritic and T-regulatory cells was studied at different time point by FACS and FISH analysis. Weight was followed as an indicator of the mice health status. The spleen weight and cellularity were followed as a sign of cytotoxicity and immune suppression. *Results.* In both groups, mice weight decreased dramatically on day 0, however the mice gained weight rapidly in group Cy-Bu compared to that seen in group Bu-Cy. The spleen weight and cellularity in group Cy-Bu reached the level of control mice faster (on day +3) compared to that found in group Bu-Cy (on day +6) indicating that the repopulation of lymphoid T and B cells in group Cy-Bu is faster than in group Bu-Cy. Chimerism reached 30% and 50% of donor cells in spleen following the Y-chromosome at day 30 and 40, respectively, in Cy-Bu group compared to 10 and 20% in Bu-Cy group. The levels of IL2 and TNF-alfa were lower at day 0 and day +1 in group Cy-Bu compared to control group while in group Bu-Cy the levels of both cytokines were about 2-fold higher compared to control. *Conclusion.* We conclude that the use of Cy-Bu compared to the traditional Bu-Cy conditioning may be beneficial for the patients since it allow faster engraftment of the stem cells. These also may help in decreasing the side effect due to the lower levels of cytokines during transplantation period.

0361

HIGH PROPORTIONS OF CD4+CD25+ CELLS IN BLOOD LYMPHOCYTES DETECTED EARLY POST HSCT ASSOCIATE WITH AGVHD AND HERALD ITS SEVERITY

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Background. CD4+CD25+ cells have been already described as regulatory cells exerting immunosupression. Unexpectly, we found that these cells are rather elevated at the beginning of aGvHD prior to steroid therapy. *Aim.*In the present study we investigated whether CD4+CD25+ cells proportions correlated with the severity of aGvHD and influenced chimerism post HSCT in patients receiving non-myeloablative conditioning regimen. *Methods.* Forty four cases (40 hematological disease and 4 immune deficiencies) transplanted from matched sibling (19) and unrelated (25) donors were studied. Thirty three patients were on non-myeloablative and 11 on myeloablative conditioning regimen. The presence of CD4+CD25+ cells in addition to routine lymphocyte profiling was investigated in three time intervals post HSCT (until +30 days, 30-60 days and 60-100 days). Post transplant chimerism was detected with the use of informative genes STR alleles determination between 12'30 days post HSCT. aGvHD were diagnosed clinically and usually the diagnosis was supported by target organ histopathology including immunostaining. *Results.* Thirty four patients were investigated by one month post HSCT. Mean value±SD of CD4+CD25+ cells equaled 9.9%±7.7 with a bimodal distribution of individual results allowing dividing the entire group of patients into two subgroups with a cut-off point at 5%. Fourteen and 20 patients had CD4+CD25+ cells below and above 5%, respectively. Twenty eight patients received non-myeloablative conditioning and they were evaluated for the early post-transplant chimerism. Seven out of 16 and 3 out of 12 patients had mixed chimerism in patients groups with high and low proportions of CD4+CD25+ cells. Twenty nine patients receiving myelo- or non-myeloablative conditioning developed aGvHD. aGvHD patients had higher proportions of CD4+CD25+ cells in blood lymphocytes detected at the beginning of aGvHD than those lacking aGvHD and investigated at the similar time post transplant (11,0±1,5 vs 3,7±0,7 respectively, p=0,000007). In addition we found that the level of CD4+CD25+ detected at the beginning of aGvHD correlated with the severity of this complication being full blown at some time post CD4+CD25+ cells measurements. The proportions of CD4+CD25+ cells were 8,4%±1,2 and 12,5%±2,5 in pts having grade I and more severe aGvHD, respectively. The highest proportions of CD4+CD25+ cells in blood lymphocytes were found in patients with

grade IV aGvHD and these values were significantly higher as compared to grade I aGvHD cases (15,2%±2,7 vs 8,4%±1,2 for grade IV and I of aGvHD, respectively, p=0,05). *Summary.* It appears that higher proportions of CD4+CD25+ in blood lymphocytes measured soon after HSCT tended to be associated with mixed chimerism but importantly associated with early manifestation of aGvHD and heralded a severe course of this complication.

0362

SHORTENING OF NEUTROPENIA IN LYMPHOMA PATIENTS AFTER TRANSPLANTATION OF LIN ENRICHED CELLS EXPANDED *EX VIVO*

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Background. Hematopoietic stem cells are able to regenerate hematopoiesis in all lineages. They are clinically used in transplantation of bone marrow or peripheral blood stem cells (PBSC) after myeloablative regimens of chemotherapy in the patients with diagnosis of leukemia or lymphoma. *Aims.* The methods of enrichment, isolation, cultivation and expansion of hematopoietic stem cells open the way for specific cellular therapy. In this study, the influence of *ex vivo* expanded Lin' enriched stem cells on the speed of engraftment was evaluated. *Methods.* Authors analyzed expansion of hematopoietic stem cells (HSC) selected by immunomagnetic separation of Lin' cells in the culture of serum-free medium *in vitro* with combination of 5 cytokines (SCF, Flt-3-L, IL-3, IL-6, G-CSF). Cell counts, morphology, immunophenotyping, S-phase, electron microscopy, and biological tests of LTC-IC, CFU-GM and CFU-Meg were analyzed. Clinical protocol was designed based upon these *Results.* Hematopoietic stem cells were enriched from apheresis products collected from patients undergoing mobilization chemotherapy by Lin' separation and expanded *in vitro*. Clinical transplantation protocol based on these results was developed. 10 patients with diagnosis of Hodgkin's or non-Hodgkin's lymphoma indicated for high-dose chemotherapy and autologous PBSC transplantation were enrolled to the protocol. All patients underwent standard PBSC collection, BEAM chemotherapy regimen from day -7 and autologous transplantation at day 0. Besides that, an extra PBSC graft was collected, hematopoietic stem cells were enriched by Lin' procedure and cells were frozen. At day '14, enriched cells were thawed and cultured in the presence of 5 cytokines in serum-free medium. Expanded cells were infused at day 0 to the patients at the escalating dose from 5.10^7 to 3.10^9 cells. Patients were closely monitored, side effects and time to engraftment in leucocytes and platelets was observed. The results were compared to historical controls of 143 patients with diagnosis of lymphoma transplanted with identical BEAM regimen and PBSC grafts. *Results.* Isolated Lin' cells in culture differentiate, the relative proportion of CD34+ cells decreases below 5% at day +14. Growing number of granulocytic progenitor cells correlates with number of CFU-GM colonies. The highest number of CFU-GM colonies and total cell expansion was observed at day +14 in cytokine combination SCF+IL-3+FLT-3-L and IL-6, which was used in the clinical protocol. The procedure of Lin' cells transplantation was free of side effects in all patients. Engraftment in leucocytes occured from day +6 to day +9 in the study group. Compared to historical controls, there was a significant shortening of neutropenia to 5.6 days in average and to 5.0 days in patients who received doses over 1.10^9 cells. There was no significant change in the engraftment in platelets (day +10 versus day +11). *Conclusions.* Hematopoietic stem cells can be enriched from PBSC grafts, cultured and expanded *ex vivo*, and safely used in the cellular therapy protocols. At higher doses of infused cells, the procedure resulted in shortening of critical period of pancytopenia.

This work was supported by grant IGA NR/8003-3.

0363

CONTINUOUS INFUSION IDARUBICIN AND ORAL BUSULPHAN (IBU) AS CONDITIONING FOR PATIENTS WITH ACUTE MYELOID LEUKEMIA UNDERGOING AUTOLOGOUS STEM CELL TRANSPLANTATION

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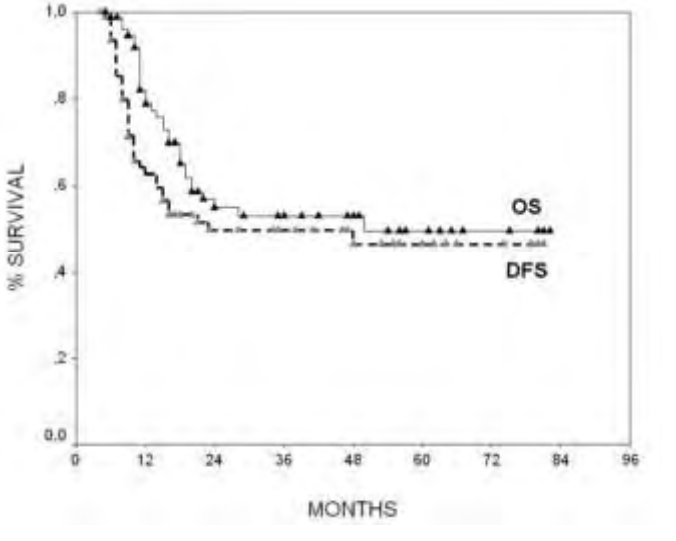
Background. One way for reducing the relapse rate after autologous stem cell transplantation (ASCT) in acute myeloid leukemia (AML) in first complete remission (CR) is the adoption of new conditioning regimens. We developed an original conditioning program, named IBu, consisting of the combination of high dose idarubicin (IDA), given at 20 mg/sqm as 3 days continuous infusion from day -13 to -11 and busulphan (Bu) at 4 mg/kg from day -5 to -2, whose feasibility was previously demonstrated in a phase II study on 14 patients (Ferrara *et al.,* THJ 2001). *Aims.* To report results from a series of 80 AML patients autografted in first CR conditioned with IBu regimen. *Patients and Methods.* There were 50 males and 30 females with a median age of 53 years (16-77). All patients had non M3-AML autografted in first CR. Karyotype was evaluable in 75 cases, with favourable, intermediate and unfavourable cytogenetics being found in 4, 60 and 11 cases, respectively. All patients received peripheral blood stem cells (PBSC) collected after consolidation plus G-CSF. The median interval between CR achievement and ASCT was 3 months (3-10). The median number of CD34+ cells infused was $6,5\times10^6$/kg (2,1-29). In patients aged more than 60 years (n=24), IDA and Bu were reduced to two and three days, respectively. *Results.* One case of transplant related death (1.2%) occurred in a patient aged 55 years, due to septyc shock. The median number of days with granulocytes <500/cmm and of platelets <20000/cmm was 10 (7-21) and 12 (6-168), respectively. The median number of platelet and blood units transfused was 3 (0-8) and 2 (0-12), respectively. Extra-hematological toxicity mainly consisted of grade WHO III-IV stomatitis (62/88 or 77%) requiring in all cases total parenteral nutrition, while 2 patients had grade III hepatic toxicity and one experienced transient hallucinations. Furthermore, most patients had FUO, while 3 experienced documented infection. Median days of intravenous antibiotics, required in 75 cases, were 11 (4-28). LVEF examination post-ASCT did not reveal any cardiac toxicity. Finally, median time of hospitalization was 28 days (22-49). At the time of writing, 43 patients (54%) are in continuous CR, while 36 have relapsed at a median time from ASCT of 5 months (1-44), with only three patients relapsing after more than one year from ASCT. One patient died in CR from gastric cancer. After a median follow-up for surviving patients of 29 months from ASCT, median overall and disease free survival are 52 months and 48 months, respectively, as shown in the figure. Patients aged more than 60 years did not experience more complications than younger patients. *Conclusions.* Our data demonstrate the efficacy of the IBu regimen in patients with AML, due to a substantial reduction of relapse rate. The most relevant toxicity of the regimen was severe mucositis requiring TPN.



0364

HIGHER DOSE OF CD4+ T CELLS IN THE ALLOGRAFT AND THE OCCURRENCE OF ACUTE GVHD ARE ASSOCIATED WITH IMPAIRED KIRS EARLY RECONSTITUTION AFTER UNMANIPULATED HLA-MISMATCHED/HAPLOIDENTICAL BLOOD AND MARROW TRANSPLANTATION

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Backgrounds. The beneficial effect of killer immunoglobulin-like receptors (KIRs) driven alloreactivity of NK cells had been proved in the T-cell-deplete hematopoitic stem cell transplantation (HSCT), but with the inconsistent effects in the T-cell-replete HSCT. These differences seemed to result from the differences in transplant protocols that utilize different extents of T cells depletion *in vitro* or *in vivo* with the existence of antithymocyte globulin (ATG). *Aims.* The goal of this study was there-

fore to address KIR (i.e. CD158a, CD158b, and CD158e) and CD94, NKG2A recovery on the NK cells after HLA-mismatch/haploidentical HSCT (with T-cell repletion). Specifically, we wished to assess any differences in KIR recovery that may affect the cytotoxity and alloreactivity of NK cells, and to compare results with those for HLA-match transplant or HLA-mismatch transplant (with T-cell depletion), as reported by Parham et al or Nguyen *et al. Results.* We sequentially evaluated 24 patients before and after HSCT on day +30, +60, +90, +120 and +180, and their donors by flow cytometry. All the patients achieved engraftment and complete donor chimerism after transplantation. All patients were alive and CCR, except 5 who died of transplant-related complications after HSCT; three patients relapsed on days 370, 330, 270 respectively. The recovery of CD94, CD94:NKG2A, CD158e (KIR3DL1) on NK cells in recipients increased first compared with their donor values on day 30 after HSCT (p=0.013, p<0.0001, and p=0.063, respectively), then sequentially decreased from day 60 to day 180, to the donor values. By day 180, NKG2A expression on NK cells was still maintained at higher levels compared with their donors' values. The kinetics of reconstitution of CD158a and CD158b(KIR2DL) was opposite to the kinetics of CD158e recovery, diminishing significantly by day 30 in patients after HSCT compared with their donor values (p=0.016 and p<0.001 respectively), then sequentially increasing by days +60 to +180 after HSCT. However, the kinetics of reconstitution of all KIRs and CD94, CD94: NKG2A on CD3+ lymphocyte after HSCT from day +30 to +180 resemble the kinetics of CD94:NKG2A recovery on NK cells after HSCT from day +30 to +180. Meanwhile, the patients were classified into *low* or 'high' CD4+ cell dose groups based on whether they received less or more than a median CD4+ cell dose of 0.85×10^8, respectively. There were a significant difference in the incidence of II-IV acute graft-versus-host-disease (GVHD) between the two groups, p=0.0226. NK cells expressed less KIRs in recipients with II-IV GVHD or receiving 'high' CD4+ cell dose compared with those with 0-I aGVHD or receiving 'low' CD4+ cell dose by day 30/60 after HSCT. Furthermore, the dose of CD4+ cells inversely correlated with the KIRs (CD158a, CD158a+CD158b+, CD158e) expression on NK cells by day 30 and 60. *Summary/Conclusion.* These results suggested that both of the T cells in grafts and the occurrence of aGVHD affected the KIRs early reconstitution on NK cells *in vivo* after HSCT.

0365

EARLY AND LONG-TERM ENGRAFTMENT AFTER AUTOLOGOUS PERIPHERAL STEM CELL TRANSPLANTATION IN ACUTE MYELOID LEUKEMIA PATIENTS

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Background. Autologous peripheral stem cell transplantation (PBSCT) has been increasingly performed in acute leukemia patients without an HLA-matched related donor; the use of mobilized PBSC has resulted in more rapid but equally effective engraftment compared with bone marrow stem cells. However, there has been considerable debate concerning which cells are related to early and long-term hematopoietic reconstitution after PBSCT. Two populations of CD34+ hematopoietic stem cells, namely pluripotent or *immature* stem cells (with expression of CD90 and lack of expression of CD38, DR, CD33) and committed or *mature* stem cells (with expression of CD38, CD33, DR and lack of expression of CD90) have been evaluated. Aim. This study aimed to identify which subset of CD34+ cells might be the most predictive of early and long-term hematopoietic recovery following autologous peripheral blood stem cell transplantation (PBSCT) in adult acute myeloid leukemia (AML) patients. *Methods.* The relationships between the number of *mature* subsets of CD34+ cells (CD34+/CD33+, CD34+/CD38+, CD34+/DR+ and CD34+/CD90-) and *immature* subsets of CD34+ cells (CD34+/CD33-, CD34+/CD38-, CD34+/DR- and CD34+/CD90+) and early and long-term hemoglobin, neutrophil and platelet counts were studied in a homogeneous series (for disease, pre-transplant chemotherapy, mobilization chemotherapy, conditioning regimen) of 29 AML patients after autologous PBSCT (14 males and 15 females aged 18-60 years, median 38). Before transplantation all patients received one induction-chemotherapy cycle (cytarabine, etoposide and daunorubicin), one consolidation-mobilization chemotherapy cycle (cytarabine and daunorubicin) and the same conditioning regimen (busulphan and cyclophosphamide). Because of the greater fragility of *mature* CD34+ compared to 'immature' CD34+ cells, the subsets of CD34+ cells were evaluated before cryopreservation and after thawing but only the cell counts after thawing were used for the correlation with early and long-term engraftment. *Results.* The most important CD34+ subset predicting early engraftment was the CD34+/CD33+ cell number, that was inversely correlated with the days to recovery of 0.5 x 109/L neutrophils (r = -0.65, p=0.05) but this correlation was weaker than the total CD34+ cells dose and early neutrophil engraftment (r=-0.71, p<0.05). The number of CD34+/CD38- cells infused correlated with the neutrophils (r=0.88, p<0.005) and platelets counts (r=0.67, p<0.05) at 12 months after PBSCT; this correlation was better than that for the total CD34+ cell dose at 12 months (r=0.36, p=0.09 for neutrophil count and r=0.48, p=0.06 for platelets count). The number of CD34+/CD90+ cells was also correlated with the platelets count at 6 (r=0.70, p<0.05) and 12 months (r=0.80, p=0.005) after PBSCT; this correlation was better than the total dose of CD34+ cells at 6 (r=0.31, p=0.3) and 12 months (r=0.48, p=0.06) for the platelets count. *Conclusions.* We suggest that the study of CD34+ cells subsets is useful for the evaluation of long-term hematopoietic reconstitution after autologous PBSC transplantation and that the cell doses of CD34+/CD38- and CD34+/CD90+ are good predictors of neutrophils and platelets long-term engraftment. In the clinical setting, in patients with a borderline or suboptimal CD34+ cell dose, measurement of CD34+/CD90+ and CD34+/CD38- cell numbers may provide additional information about the graft quality.

0366

MURINE MODEL OF STEM CELL TRANSPLANTATION AND GVHD BASED ON CHEMOTHERAPY CONDITIONING

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Background. Stem Cell Transplantation (SCT) is a curative treatment for a wide range of malignant and non-malignant diseases. In spite of its benefits to the patients, there are numbers of obstacles limiting the wide use of SCT. Because of ethical and technical problems, animal models of SCT are used widely to study basic mechanisms underlying SCT and SCT-related complications such as veno-occlusive disease (VOD) and graft versus host disease (GVHD). The majority of mice models of SCT and GVHD are based on the use of radiotherapy as a conditioning regimen. However, these models can not cover the variety of SCT in clinical settings. Many patients are conditioned with chemotherapy which may affect the occurrence and rate of transplantation related complications. *Aims.* To establish a murine model of SCT and GVHD using the chemotherapy as conditioning regimen. *Methods.* One hundred and twenty female BALB/c mice were divided in two main groups. Group I considered for syngeneic SCT and group II considered for allogeneic SCT. Each group was divided into two subgroups. Cy-Bu subgroups received Cy (100 mg/kg/day for two days) followed by liposomal Bu (15 mg/kg/day four days). Bu-Cy subgroups received the same dose of the drugs but in reversed order. Forty two of the recipients (group I) were transplanted by bone marrow stem cell (Sca-1) of male BALB/c (syngeneic) and forty two recipients from group II were transplanted by bone marrow stem cell (Sca-1) of male C57BL/6 (allogeneic). The chimerism and engraftment were surveyed by FISH analysis. Cytokine levels and immune cell repopulation and dynamics were studied by FACS analysis. *Results.* Engraftment was established in both groups successfully and started from day +15. Also there were differences in time period of engraftment. In allogeneic group we could show the occurrence of GVHD as well. GVHD has shown symptoms of acute GVHD and occurred between day + 30 and day + 40 post transplantation. Interestingly this conditioning is not myeloablative and can consider as non-myeloablative conditioning model of SCT. *Summary/Conclusion.* We have established a new murine model of SCT using chemotherapy which is compatible and comparable with non-myeloablative model of conditioning in human. This model can also be used to study the basic mechanisms underlying GVHD that might be caused by the effect of the conditioning regimen on different cell sub-populations.

0367

IN VIVO LUCIFERASE EXPRESSION OF TRANSDUCED HEMATOPOIETIC STEM CELL POPULATIONS USING THE NON-INVASIVE BIOLUMINESCENT IMAGING

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The homing and outgrowth of luciferase gene-transduced hematopoietic cells can be visualized in live animals on sequential time points by bioluminescent imaging (BLI), using a highly sensitive liquid nitrogen cooled charge-coupled camera (CCCD). A safe transduction of bone marrow (BM) hematopoietic cells was optimised for the retroviral vector encoding the Green Fluorescent Protein-luciferase (GFP-Luc) fusion gene. To validate the signal of the BLI in relation to the number of transduced hematopoietic cells, different cell doses were transplanted. There is a linear correlation of the number of cells and the bioluminescence signal in the BM compartment during the first 5 weeks after transplantation. However, after a longer period of time the variation increased between the individual mice. Studying the fate of different transduced murine HSC populations after transplantation into lethally irradiated mice, not-treated BM was compared to Sca-1 positive cells from 5Flurouracil-BM, a technique to enrich for the primitive hematopoietic stem cell. After transplantation of the total cell population with 20% transduced cell, different foci in the BM showed luciferase activity, predominantly in the femurs and sternum. Luciferase activity in mice transplanted with transduced BM cells decreases below detection level after 6 weeks, suggesting that only committed progenitors were transduced in this cell sample. Mice transplanted with transduced Sca-1 positive cells reached a maximum level of luciferase expression at week 4-5 and thereafter a consistent signal during the 7 months, indicative for the activity of the transduced primitive stem cells. The transduction of primitive stem cells was confirmed by a secondary transplantation in which long-term expression of luciferase was observed. These results show that the BLI might be of value to study different populations of hematopoietic stem cells or for monitoring and quantitate the proliferation of locally active hematopoietic cells.

Infectious diseases (including supportive care)

0368

RELATIONSHIP BETWEEN HTLV-1-ASSOCIATED ANTIBODIES, TAX-SPECIFIC CYTOTOXIC T LYMPHOCYTES, AND PROVIRAL LOAD AMONG HTLV-1 CARRIERS

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Background. Previous studies have demonstrated that higher anti-HTLV-1 antibody titer and lower anti-Tax antibody titer in human T-lymphotropic virus type 1 (HTLV-1) carriers imply a higher HTLV-1 proviral load and greater risk of ATL. However, it is still not fully understood how these factors are correlated with each other in HTLV-1 carriers. *Aims.* The present study was performed to examine the relationship among anti-HTLV-1 antibody, anti-Tax antibody, Tax-specific CTLs, and proviral load to clarify the significance of these factors. *Methods.* Forty-five HTLV-1 carriers were examined. Anti-HTLV-1 antibody was measured by ECLIA and anti-Tax antibody was measured by ELISA. Sixteen distinct HLA-A*0201 and HLA-A*2402 tetramers were prepared to detect HTLV-1 Tax or Env epitope-specific CTLs. Quantification of HTLV-1 DNA was performed by real-time PCR in a Light-Cycler System. *Results.* There were significant positive correlations between the frequency of Tax301-309-specific CTLs and both anti-HTLV-1 titer (r = .549, p=.001) and anti-Tax titer (r = .505, p=.003), whereas the frequency of Tax11-19-specific CTLs was correlated with neither antibody titer. However, both the frequency (median 0.14% vs. 0.00%, p=.002. Mann-Whitney U test) and the prevalence (100% vs. 30.8%, p=.029. Fisher's exact test) of Tax11-19-specific CTLs in the anti-Tax-positive group were significantly higher than those in the negative group. The frequency (median 0.19% vs. 0.09%, p=.033) of Tax301-309-specific CTLs in the anti-Tax positive group was also significantly higher than that in the negative group, whereas the prevalence of the CTLs (84.6% vs. 60.0%, p=.245) was not different between the two groups. The proviral load ranged from 4.6 to 592.4, with a median value of 62.3/1000 copies. The proviral load in the Tax11-19-specific CTL positive group was significantly lower than that in the CTL negative group (median 24.1 vs. 69.5, p=.017), although no difference was observed between the Tax301-309-specific CTL-positive and negative groups (median 69.0 vs. 33.5, p=.291). However, multivariate regression analysis showed a positive correlation between anti-HTLV-1 titer and proviral load (r = .510, p=.001), and negative correlations between HLA-A*0201 positivity and proviral load (r = _.413, p=.004) and between the frequency of Tax301-309-specific CTLs and proviral load (r = _.413, p=.007), whereas HLA-A*2402 positivity and the frequency of Tax11-19-specific CTLs had no independent effect on HTLV-1 proviral load. *Summary/Conclusions.* Anti-Tax titer, the frequency of Tax301-309-specific CTLs, and HLA-A*0201 positivity (which confers efficiency to the Tax11-19-specific CTL response) may prevent growth of HTLV-1-infected cells in HTLV-1 carriers, whereas higher anti-HTLV-1 titer is a risk factor for higher HTLV-1 proviral load.

0369

WHAT IS THE VALUE OF THE NECROPSIC STUDY ON CLINICAL HISTORY OF PATIENTS WHO DIED OF MALIGNANT HEMATOLOGIC ILLNESSES?

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Background. During decades the autopsy has been important in order to complete clinical expressions understanding for clinical manifestations knowledge. In the last two decades many outstanding advances on diagnostic procedures of malignants hematologic illnesses had taken place (new image tests, new techniques like flow cytometry, molecular biology, microbiology and biological markers). *Aims.* It is necessary to wonder if the necropsic study continuous being worthy for the comprehension of the clinical profile presented by patients affected of malignants hematologic illnesses. *Patients and Methods.* We have compared the available clinical evidences in the clinical history and the results of the necropsic study in 24 patients with Acute Leukemia and 10 patients with other hematologic neoplasias that had undergo autologous progenitor cell transplantation. *Results.* The necropsic study provide contributed data that didn't figure in clinical valuation: 1) opportunist infection for

a not suspected germ in 3 patients (Tuberculosis, Citomegalovirus, Aspergillus).; 2) erroneous interpretation of the final symtoms etiology in different aspects: we suspect opportunist infection that was not confirmed in the autopsy, and that was turn into leukemic infiltration (2 cases); compatible clinic with gastrointestinal acute graft-versus-host disease that change to leukemic infiltration (1 case) or fungal infection (1 case). Finally, the autopsy disclosed unexpected involvement of different organs by opportunist infections in 10 patients. *Conclusion.* In spite of the advance on the diagnostic procedures, we confirm the profitability of the autopsy as source of valuable information for the clinical manifestations in patients with malignants hematologic illnesses.

0370

CMV INFECTION AND DISEASE AFTER ALLOGENEIC STEM CELL TRANSPLANTATION A SINGLE CENTER EXPERIENCE

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Background/Aims. CMV infection and disease remains a significant cause of morbidity and to some degree mortality after allogeneic stem cell transplantation (ASCT). The aim of this retrospective study was to evaluate the frequency of CMV infection and disease after ASCT at our center and to identify possible risk factors. *Patient/Material/Methods.* From 1994 to 2004 330 patients underwent ASCT. Conditioning regimens were oral Busulfan 16 mg/kg and iv Cyclophosphamide 60 mg/kg (Bu4Cy2) (N=297) or Cyclophosphamide and ATG (N=10), 23 patients received other conditioning regimens. Graft versus host disease (GvHD) prophylaxis was cyclosporine A (CyA) and a conventional short course of Methotrexate. Established acute GvHD ≥ grade II was treated with increased immunosupression with CyA and corticosteroids, occasionally ATG and other immunosuppressants in steroid refractory GvHD. Blood products were leukocyte depleted or CMV negative to CMV negative recipients. CMV surveillance was routinely performed by measurement of pp65 or PCR 1-2× / week until tapering of immunosupression. CMV infection was diagnosed by detection of matrix protein pp65 or CMV DNA by PCR in two consecutive samples, which prompted treatment with ganciclovir or foscarnet. CMV disease was diagnosed according to the criteria of the 4. International Cytomegalovirus Workshop, Paris, 1993. *Results.* CMV infection was diagnosed in 22% (N=73) and CMV disease in 6% (N=19). Gastrointestinal (GIT) CMV disease was diagnosed in 11; lung in five, lung and GIT in two and spleen in one case. Two CMV infections were primary, whereas primary CMV disease was not observed. Of the patients who were CMV negative pretx (N=95); infection developed in 3 in patients receiving grafts from CMV positive donors. In the group of patients receiving grafts from family donors (n=220) the incidence of CMV infection and disease was 18.6% and 4% respectively. In the group receiving grafts from matched unrelated donors (MUD) (n=110) these incidences were 29% and 9%. CMV infection and disease were diagnosed at a median of 43 and 48 days post transplant. Late CMV infection, after day 100, was diagnosed in 9 patients. Using logistic regression analysis, the following factors were found to be statistically significant for development of CMV infection and disease: graft from MUD, use of corticosteroids, and CMV seropositive recipient pretx. Mortality in patients with CMV infection and disease was 47% and 68%, compared to 44% in the total material. *Conclusions.* In this retrospective single center study the incidence of CMV infection and disease was 22% and 6%. The low incidence of infection might be due to the fact that two positive tests and antiviral treatment were required for diagnosis. Grafts from MUD, use of corticosteroids and seropositive recipient pretx were identified as risk factors. Based on autopsies, CMV disease did not seem to be the direct cause of death, but the overall mortality is high, reflecting the severity of immunosupression.

0371

AMPLIFICATION OF AN ASPERGILLUS SPP. SPECIFIC REGION OF THE 18S RRNA GENE BY REAL-TIME POLYMERASE CHAIN REACTION ON SERUM SAMPLES AS DIAGNOSTIC TOOL FOR INVASIVE ASPERGILLOSIS IN FEBRILE HEMATOLOGICAL PATIENTS

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Background. Invasive aspergillosis (IA) is responsible for about 5-10% cases of fever of unknown origin during neutropenia in hematological cancer patients. The diagnosis of invasive aspergillosis is conventionally based on indirect criteria devised by international cooperative study groups (EORTC-MSG) which include the determination of galactomannan (GM) antigenemia. However the precise cut-off values of GM antigenemia are still debated and the sensitivity and specificity are still suboptimal. DNA-based methods have shown potential utility in the diagnosis of invasive fungal infections and they may be a valid alternative to GM test. *Aims.* We retrospectively evaluated a new Aspergillus specific real-time polymerase chain reaction (AspRT-PCR) test in the serum of patients affected by hematological malignancies at the onset of fever, in order to evaluate the correlation between the result of AspRT-PCR with the subsequent clinical diagnosis. *Methods.* Twenty-three patients affected by acute leukemia (n=9), lymphoma (n=9), myelodysplastic syndrome (n=2), chronic lymphocytic leukemia (n=2) were evaluated. They underwent a complete microbiological screening, chest radiograph and CT scan. GM antigenemia >1 was considered positive. AspRT- PCR was performed with an Aspergillus gene-specific Taqman probe and Applied Biosystems 770 instrument. The Aspergillus-specific probe was designed using Primer Express software, within a conserved region of the 18S rRNA gene of A. fumigatus, A. flavus, A. nidulans, A. niger, A. terreus, A. versicolor, but not homologous to other sequenced pathogenic fungi or mammalian DNA. Thirty sera obtained from healthy voluntaries were evaluated as control. *Results.* All the healthy voluntaries were found negative and no cross amplification was observed. According to the EORTC-MSG diagnostic criteria, we classified 5 patients as having *probable* aspergillosis, 8 as *possible* aspergillosis and 10 as *no* aspergillosis. GM antigenemia was positive in 3/5 patients with *probable* aspergillosis and in 1/18 patients with *possible/no* infection. AspRT-PCR was positive in all the patients (5/5) with probable infection and in 3 of the 8 patients with possible aspergillosis. Two of the patients classified as no aspergillosis showed a positivity of RT-PCR. Considering the groups of *probable* infections as true infection and *possible/no* infection as true negative, the frequency of AspRT-PCR was significantly higher among truly infected (p=0.0075). The sensitivity and negative predictive value of the test were 100%. Specificity was 72% and the positive predictive value 50%. In three of 5 patients with *probable* aspergillosis, RT-PCR became positive earlier than galactomannan antigen (median 5 days, range 5-9). *Conclusions.* AspRT-PCR for invasive aspergillosis showed an excellent sensitivity and negative predictive value. Moreover, it could be an earlier marker of fungal infection in comparison with GM antigen test, although these results have to be confirmed on larger number of patients. Further studies are needed to disclose factors accounting for false positive results.

0372

INFECTIOUS COMPLICATIONS IN HAEMATOLOGICAL PATIENTS WITH CENTRAL VENOUS CATHETERS: A PROSPECTIVE ANALYSIS OF RISK FACTORS AND ETIOLOGICAL AGENTS

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Background. The use of central venous catheters (CVCs) in haematological patients is associated with various complications, among which infections are the most frequent and life-threatening. *Aims.* The aim of this single-centre, prospective study was to evaluate the epidemiology and the outcome of catheter-related infectious complications in haematological patients. *Methods.* Data concerning catheterizations of patients with haematological malignancies were collected between September 2002 and December 2004. The study cohort included 279 patients (137 male and 142 female, mean age 49.7, range 17-75) for a total of 388 catheterizations: 120 acute myeloid leukaemia (43%), 24 acute lymphoid leukaemia (8.6%), 70 lymphoma (25.1%), 44 multiple myeloma (15.8%), 21 with other haematological malignancies (7.5%). In acute leukaemia pts CVCs were used for chemotherapy administration and support therapy during aplasia, while the principal use in lymphoma and multiple myeloma pts was to harvest peripheral blood stem cells. A catheter-related bloodstream infection (CR-BI) was defined by demonstration of the same microrganism both in the catheter and in the peripheral blood cultures, when no other source of infection other than the catheter itself was found. *Results.* Mean duration of catheterization was 18.8 days, while mean neutropenia with ANC < 0.5×10^9/L during catheter *in situ* maintenance was 7.4 days and mean severe neutropenia with ANC < 0.1×10^9/L was 4.7 days. In particular, in acute leukaemia pts mean duration of catheterization was 23.8 days, mean neutropenia with ANC < 0.5×10^9/L was 11.3 days, mean neutropenia with ANC < 0.1×10^9/L was 7.3 days. Exit tunnel infections occurred in 19 cases (2.6 per 1000 catheter days), while catheter-related bloodstream infections

occurred in 49 cases (6.7 per 1000 catheter days). Gram-positive CR-BIs were 69%, among which Staphylococcus epidermidis and Streptococci were prevalent (58% and 12%, respectively). The remaining were Gram-negative CR-BI, most of which caused by E.coli, Pseudomonas aeruginosa and Enterobacter spp (31%, 23% and 15%, respectively). No fungal CR-BI was diagnosed. During hospitalization two patients (0.7%) died due to their haematological disease; catheter removal because of infectious complications was necessary in 14 cases (3.61%), of which 6 showed CR-BI. At univariate analysis, significant risk factors for CR-BI were number of days/catheter (p<0.0001), chemotherapy dose (high vs. standard dose; p<0.015), duration of neutropenia (p<0.001) and thrombocytopenia (p<0.001). At multivariate analysis, only days/catheter and duration of neutropenia appeared significant risk factors for CR-BI. *Conclusions.* The incidence of CR-BI is greatly increased by risk factors connected to haematological diseases and consequent chemotherapy administration, such as duration of catheterization, neutropenia and thrombocytopenia. In particular, patients affected by acute leukaemia are at a higher risk for CVC-related infections due to the use of aggressive and/or high-dose chemotherapy cycles during induction and salvage therapies, and a longer duration of severe neutropenia.

0373

RESPIRATORY VIRAL INFECTIONS IN IMMUNOCOMPROMISED PATIENTS

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Background. Acute respiratory viral infections are generally self limiting, but can lead to morbidity and mortality in immunocompromised patients particularly in the setting of bone marrow transplantation. Mortality in previous series has varied from 30 -100%. *Aims.* 1. To establish the incidence of respiratory viral infection in our unit. 2.To identify high risk patients requiring treatment and determine outcome. *Methods.* During the study period, July 2003-June 2005, all symptomatic patients had samples of respiratory secretions, nasopharyngeal aspirate (NPA) or bronchoalveolar lavage evaluated for respiratory viruses. Symptoms included cough, fever and coryza. Results were phoned to the referring doctor. *Results.* There were 40 positive results in thirty-eight patients. The following viruses were identified and patients were commenced on appropriate therapy ; oseltamivir for influenza A or B; Nebulised ribavarin for 5 five days and alternate day intravenous immune globulin for RSV and parainfluenza.

RSV	n =11	(29%)
PARAINFLUENZA 2	n=2	(5%)
PARAINFLUENZA 3	n=16	(42%)
INFLUENZA A	n=8	(21%)
INFLUENZA B	n=1	3%)
ADENOVIRUS	0	

There were no deaths associated with respiratory virus infection during the study period (July 2003-June 2005) One patient with chronic GvHD required non invasive ventilation for acute respiratory distress due to RSV. Two patients required a second course of treatment due to persistent symptoms. Two transplants were deferred due to Influenza A. *Conclusion.* A high index of suspicion with early investigation and prompt isolation and treatment reduces the morbidity and mortality associated with these infections in immunocompromised patients. The presence of lymphopenia, GvHD, and steroid administration are risk factors for poor outcome.

0374

REVERSE SEROCONVERSION OF HEPATITIS B VIRUS AFTER ALLOGENEIC OR AUTOLOGOUS HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Background. Reactivation of hepatitis B virus (HBV) in patients with anti-hepatitis B surface antigen antibody (HBsAb) has been known as reverse seroconversion, and recognized as a rare complication after hematopoietic stem cell transplantation (HSCT). However, its precise incidence has yet to be fully elucidated. *Aims.* We retrospectively analyzed the incidence of HBV reverse seroconversion in patients undergoing allogeneic or autologous HSCT for hematologic diseases. *Patients and Methods.* Eighty-three patients undergoing allogeneic HSCT (allo-HSCT: n=55) or autologous HSCT (auto-HSCT; n=28) between March 1992 and December 2005 were HBsAb-positive before transplant, and could be evaluated. Median age was 44 years-old (range 18-61) in allo-HSCT recipients, and 52 years-old (range 26-66) in auto-HSCT recipients. Diagnoses were acute leukemia in 25, non-Hodgkin's lymphoma (NHL) in 22, multiple myeloma in 20, chronic myelogenous leukemia (CML)in 8, myelodysplastic syndrome in 7, and aplastic anemia in 1. Stem cell sources were bone marrow or peripheral blood stem cells (PBSCs) from related donor (n=23), bone marrow (n=30) or cord blood (n=2) from unrelated donor for allo-HSCT, and PBSCs (n=28) for auto-HSCT. Only 2 of 55 allogeneic donors were HBsAb positive. For conditioning, patients received myeloablative (n=44) or reduced-intensity regimen (n=11) for allo-HSCT, and high-dose melphalan regimen (n=17), MCVAC regimen (n=9), or total body irradiation-based regimen (n=2) for auto-HSCT. *Results.* Three of 55 patients (5.5%) and 3 of 28 patients (10.7%) experienced HBV reverse seroconversion after allo- and auto-HSCT, respectively. Time to reverse seroconversion from HSCT was 7.8, 10.5, and 53.6 months in 3 allo-HSCT recipients, and 4.6, 6.1, and 6.6 months in 3 auto-HSCT recipients. Underlying diseases were acute leukemia, CML, multiple myeloma in allo-HSCT recipients, while multiple myeloma in 3 auto-HSCT recipients. In 4 (allo-HSCT 3, auto-HSCT 1) out of 6 patients, clinical hepatitis was diagnosed. *Conclusions.* HBV reverse seroconversion after HSCT is not infrequent, and close HBV monitoring is strongly recommended in HBsAb-positive patients. Furthermore, it is suggested that allo-HSCT recipients might be at higher risk of developing clinical hepatitis due to HBV reverse seroconversion than auto-HSCT recipients.

0375

HEPATITIS AND REACTIVATION OF HBV DURING TREATMENT OF DLBCL PATIENTS: AN UNDERESTIMATED EVENT

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Background. Hepatitis due to HBV reactivation after high dose chemotherapy for cancer patients is a well-recognized complication in chronic HBV carriers. The clinical consequences of hepatic injury range from asymptomatic liver dysfunction to massive hepatic necrosis and death by liver failure. *Aims.* The aim of this study is to compare the occurrence of hepatitis and HBV reactivation in Diffuse Large B Cell Lymphoma (DLBCL) patients with distinct HBV serologic pattern. *Methods.* We reviewed the medical files of sixty two patients with DLBCL followed since 1997 until now. All patients were treated according to the IPI score of the lymphoma. We defined 3 groups of patients based on the serologic HBV pattern at diagnosis. The first one included patients who had no antibodies anti-HBc and no antigen HBs (AbHBc- / AgHBs-), the second group included patients who had antibodies anti-HBc but no antigen HBs (AbHBc+/AgHBs-). The third group included patients who carried antigen HBs (AgHBs+). The endpoint of this study was the occurrence of hepatitis witch was defined by serum ALT level above 100 UI for at least three consecutive days. We also reviewed the severity of hepatitis and occurrence of HBV reactivation, defined as a raise in serum HBV DNA level or a re-appearance of AgHBe in serum. *Results.* We identified Forty one patients in the AbHBc-/AgHBs- group, thirteen in the AbHBc+/ AgHBS- group and four in the AgHBs +. None of the patients had hepatitis before treatment. Seventeen patients developed hepatitis during or after the treatment: Nine in AbHbc-/AgHBs- group (9/41 = 22%), four in AbHBc+/AgHBs- (4/13= 31%) and four in AgHBs+ group (4/4 = 100%). The rate of hepatitis was significantly higher in AgHBs+ group than in AbHBc-/AgHBs- group (100% vs 21%, p=0.001) and than in AbHBc+/AgHBs- group (100% vs 31%, p=0.015). A trend to higher rate of hepatitis development was observed in AbHBc+/AgHBs- group comparatively to AbHBc-/Ag HBs- (31% vs 21%, p=0.076). All the patients in the AgHBs+ group developed HBV reactivation and severe hepatic complications. Three of them died from liver failure. Interestingly, one patients of the AbHBc+/AgHBs- developed a HBV reactivation with acute hepatitis (ALT >1000 UI). In the others patients hepatitis was transient, ALT levels did not exceed 300 UI and no seroconversion occurred. *Conclusions.* Despite the small number of patients, we observed a significant higher risk to develop severe hepatitis in DLBCL patients with AgHBs+ (chronic HBV carriers). In those cases, hepatitis was due to HBV reactivation and associated with a high mortality. Hepatitis related to HBV reactivation occurred also in AbHBc+/AgHBs- patients, who have presumed resolved hepatitis B. These results emphasize the need for a careful follow-up for chronic HBV carriers and patients with pre-

sumed resolved hepatitis B. Prophylaxis for HBV reactivation should be administered to all chronic HBV carriers before chemotherapy regards to the high risk of severe hepatitis and HBV related death. The opportunity of viral prophylaxis in patients with antiHBc antibodies but no HBs antigen deserves to be further investigated.

0376

ORAL VALGANCICLOVIR IS AN EFFECTIVE PRIMARY PREEMPTIVE THERAPY OF CYTOMEGALOVIRUS DISEASE IN RECIPIENTS OF ALLOGENEIC STEM CELL TRANSPLANT

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Cytomegalovirus (CMV) infection is a common complication after allogeneic SCT. Valgancyclovir hydrochloride (VALCYTE-VGC) is a prodrug of ganciclovir, orally available, that has been used in CMV infection in high-risk solid organ transplants (donor positive, recipient negative); there were only a few data about this drug in allogeneic SCT. The primary aims of our study were the assessment of efficacy and safety of VGC as preemptive therapy of CMV disease after allogeneic stem cell transplantation. This study is ongoing and here we are reporting the preliminary results. During a five-month-period VGC was administered to 10 consecutive patients (pts) with a CMV infection which was diagnosed after a median time of 86 days (range 59-480) from transplant. There were 6 males and 4 females (myelofibrosis 2, leukemia 4, myeloma 2, lymphoma 2). The median age was 55 years (range 43-66); 7/10 pts underwent stem cell transplantation from unrelated and 3/10 from related donors; 7/10 pts received a reduced intensity conditioning regimen (RIC); CMV prophylaxis consisted in acyclovir in all cases. Pretransplant CMV serology showed that in 100% of cases either recipient and/or donor were positive (D+/R+ = 5/10, D-/R+= 5/10). At the onset of CMV infection 9/10 (90%) pts have an acute or chronic graft versus host disease for which were received therapy including prednisone plus other drugs. The pp65 antigenemia assay were positive in all cases with a median number of positive nuclei of 21±35. The starting treatment dosage of VGC was 900 mg twice a day and it was continued until the CMV antigenemia and PCR became negative in two consecutive samples. All 10 cases obtained a clearance of antigenemia after a median of 8 days of VGC therapy (range 5-16 days); viremia became negative in all cases. The median length of manteinance therapy with VGC (900 mg once-daily) was 21 days (range 8-32). Only one patient developed a mild deterioration of renal function that required dose adjustment (VGC 450 mg once-daily). None of the pts developed gastrointestinal disorders; mild anemia was reported in 3/10 (30%) pts, neutropenia in 5/10 (50%) pts and thrombocytopenia in 4/10 (40%). *Conclusions.* 1)Preemptive therapy with VGC after related and unrelated allogeneic SCT seems to be safe and effective (with a rapid clearance of antigenemia and viremia). 2)The simple once or bi-daily VGC regimen can improve the compliance of the pts. 3)Regular blood counts should be performed to early detect cytopenia. 4)The optimal dose and duration of VGC therapy in this setting need to be established with additional prospective studies.

0377

BAL AS DIAGNOSTIC TOOL IN SEVERE PNEUMONIA IN PATIENTS WITH HEMATOLOGICAL MALIGNANCIES: SINGLE CENTER REPORT ON 16 PATIENTS

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Pneumonia is one of the most frequent life-threatening complications in patients affected by hematological malignancies despite recent improvements in support therapy; in these patietns a timely identification of the microbiological agent is crucial. The aim of this study was to evaluate the utility of bronco-alveolar lavage (BAL) in etiological diagnosis of pulmonary infiltrates in this setting. Over 2 years period 16 patients affected by hematological malignancies (2 Myeloma, 1 Essential Thrombocytemia, 5 Non Hodgkin Lymphoma,4 Acute Myeloid Leukemia, 1 Myelofibrosis, 2 Chronic Lymphocitic Leukemia,1CML BC) age 38-76 years, showing clinical and radiological signs of Severe Pneumonia and failing to respond to antimicrobial therapy were studied. Together with initial routine serological and microbiological diagnostic tests on blood, urine and sputum (if available) bronco-alveolar lavage (BAL) was performed to identify the etiological agent. According to ATS (American Thoracic Society) criteria a bacterial cut-off > 10 4 CFU/mLor the isolation of a pathogen which doesn't ordinarily colonize the upper respiratory tract (M. tubercolosis, Pneumocistis J, Legionella sp, Aspergillus sp) defined infectious pneumonia. *Results.* the final diagnosis obtained by means of BAL among the 16 patients enrolled was: a) infectious pneumonia in 7 patients: the etiological agent was 2 polimicrobial infections (Micobacterium T plus E.Coli, MycobacteriumT plus Pneumocystis J), 2 Aspergillus sp (1 diagnosed by galactomannan detection on BAL and serum), 1 MRSA, 1 Corynebacterium sp. b) non infectious lung disease in 6 patients with alternative diagnosis: 2 alveolar drug damage, 2 BOOP, 1 T cell lymphoma, 1 bronchial infiltration of CLL c) 3 unknown diagnosis. Routine laboratoristic results were diagnostic only in one case (serum galactomannan detection) In conclusion: discrimination between infectious and non infectious diseases that mimic pneumonia is laborious namely in hematological patients; in our experience BAL procedure had a substantial impact on the etiological diagnosis and allowed a change of therapeutic strategy in 10 of 16 cases (62%).

0378

SURVIVAL AND DISEASE COMPLICATIONS OF THALASSEMIA MAJOR - 14 YEARS EXPERIENCE AT KING ABDULAZIZ UNIVERSITY HOSPITAL, JEDDAH, KSA

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Background. Treatment of thalassemia major is complex, expensive and requires a multidisciplinary approach. Optimal clinical care is demanding and expensive but achievable. In spite of medical treatment improving dramatically, complications and deaths still occur. *Aim.* To assess the prevalence of survival and disease complications among patients with thalassemia major at our center. *Methods.* A retrospective chart review was done of all patients diagnosed as Thalassemia Major (TM) between 1990 and 2004. The patients were followed and treated at King Abdulaziz University Hospital (KAUH), an academic tertiary care medical center. All 360 patients (203 males & 157 females) were transfusion dependant since early childhood and treated with parenteral Deferoxamine. Approximately 98% were β-TM and 2% were HbE_o. The data had been collected by means of specially prepared forms (from Hematology Clinic, Day Care and Medical Records Department). The mean of serum ferritin has been available for all patients yearly. Comparison of ferritin levels between groups was performed by Student's t test. *Results.* Out of 360 patients, 293 (81.4%) patients were alive, 27 (7.2%) patients had died, 15 (4.2%) patients underwent BMT and 25 (6.9%) patients' follow-up were lost. Twelve (3.3%) patients died from heart disease. 7 (1.9%) patients died from infections, all patients were splenectomised. The serum ferritin levels for patients who died were significantly higher than for those patients who survived (7,500 vs. 3,200; $p<0.001$). *Conclusions.* Cardiac constitutes the first important cause of death followed by infection. Infection among thalassemics is still a risk factor which needs to be addressed carefully. Splenectomized thalassemic patients required special attention to avoid and prevent fatal infections. Complications and deaths among thalassaemics are iron related organ dysfunction and age related. The majority of patients were on non-optimal chelation therapy and non-compliance. Poor compliance with parental chelation started at the adolescent age. Prevention program of inherited blood diseases should be implemented as a priority in the region.

Table 1.

Causes of Death	*Number*	*Percentage*	*Mean Age*	*Age Range*	*Mean S. Ferritin (ng/mL)*
Cardiac	12	3.3	20	16-24	7500
Infection	7*	1.9	14	10-18	2400
Endocrine	3	0.8	24	21-25	6000
Liver disease	2	0.5	21	20-22	4000
Thrombosis/bleeding	1	0.2	17	17	3500
Unknown	2	0.5	19	18-20	3000
Total	27	7.2			

**Splectomized patients.*

0379

EFFECTIVENESS OF NONINVASIVE VENTILATION IN TREATMENT OF ACUTE RESPIRATORY FAILURE IN ONCOHEMATOLGICAL PATIENTS

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Background. Use of noninvasive ventilation has been recommended in the inmunosupressed patients to avoid the endotracheal intubation complications. *Aims.* Analyses the effectiveness and safety of noninvasive ventilation (NIV) in the treatment of acute respiratory failure (ARF) in oncohematological patients. *Methods.* Observational prospective study including patients affected of disseminated malignant neoplasias admitted in our intensive care unit (ICU) because ARF and treated with NIV. Success of NIV was defined as avoidance of endotracheal intubation and survival to ICU. The quantitative variables were expressed as means±standar desviation and qualitative ones like percentage. After univariated analysis, achieves multivariate analysis by logistic regression. *Results.* In the period between january 1997 and september 2005 were admitted 135 patients with disseminated neoplasia and ARF. The mean age was 62±17 years and 65.2% was males. The malignant etiology was: 68 patients with metastatic solid organ neoplasia, 30 patients with leukemia, 24 patients with lymphoma and 13 with myeloma. Pneumonia was the most frecuent cause of ARF (31 patients, 23%) and next acute respiratory distress syndrome (29 patients, 21.5%). Do not intubation order was indicated in 63 patients (46.7%). The majority of patients were ventilated with VISON® ventilator (92.6%) and the remains patients with BIPAP STD® ventilator. BIPAP ventilation mode was used in 96.3% and CPAP mode in the rest patients. Initial ventilator pressure levels were IPAP of 15±2 and EPAP of 7±1 cms of H2O. Respiratory parameters before and after one hour of NIV therapy were, respectively: pHa: 7.32±0.11 and 7.34±0.7 (p:0.003); PaO2/FiO2: 143±37 and 168±43 (p<0.001); respiratory frecuency: 36±5 and 32±5 bpm (p<0.001). Complications due to NIV were present in 52 patients (38.5%): naso-frontal cutaneus injury (36.3%); ocular irritation (5.9%); claustrophobia (3.7%); nosocomial pneumonia (3.7%); vomiting (3%); neumothorax (1.5%); aspiration pneumonia (0.7%). Duration of NIV therapy was 2.8±2.5 days and 40.5±38.3 hours. The success of NIV was 56.3% and hospital mortality was 56.3%. The length of ICU and hospital stays were 8.4±10.2 and 2.7±16.8 days, respectively. The variables associated to NIV failure were: PaO2/FiO2 before NIV begining (OR: 0.902, CI-95%: 0.845, 0.964; p:0.002), PaO2/FiO2 one hour after NIV (OR: 0.886, CI-95%: 0.827, 0.949; p: 0.001), respiratory frecuency one hour after NIV (OR: 1.874, CI-95%: 1.274, 2.759; p: 0.001) and highest SOFA (Sepsis Organ Failure Assessment) score (OR: 1.751, CI-95%: 1.305, 2.49; p<0.001). The variables associated to hospital mortality were NIV unsuccessful (OR: 8.566, CI-95%: 2.467, 29.770; p:0.001) and highest SOFA score (OR: 1.188, CI-95%: 1.049, 1.345; p: 0.007). *Conclusions.* NIV treatment in oncohematological patients has high success rate. The patients who present NIV unsuccessful and severe multiorgan failure have worse outcome.

0380

INCIDENCE AND RISK FACTORS OF INVASIVE FUNGAL INFECTIONS IN 246 PATIENTS UNDERGOING RELATED OR UNRELATED ALLOGENEIC BONE MARROW TRANSPLANTATION.

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Introduction. Allogeneic bone marrow transplantation (BMT) is increasingly used to treat hematologic diseases. Invasive fungal infections (IFI) remain an important cause of morbidity and mortality in this setting. *Patients and Results.* To evaluate the epidemiology, outcome and risk factors of proven or probable IFI in allogeneic BMT recipients, we retrospectively examined the medical records of 246 consecutive adult patients (pts) who underwent allogeneic BMT (150 from related and 96 from unrelated donor) at our Department between 1992 and 2004; 193/246 (78%) pts received a myeloablative conditioning regimen and 53/246 (22%) a non myeloablative one. The median age of patients was 42 years (range 19-66). We identified 31 cases of IFI with an overall incidence of 13%; the incidence after related BMT (R-BMT) was 8% (12/150) while it was 20% (19/96) after unrelated BMT (UR-BMT) (p= < 0,05). The incidence was the same in the myeloablative (24/193, 12%) and non myeloablative (7/53, 13%) setting. IFI occurred after a median of 41 days from BMT (range 5-1440). There were 28 cases with proven or probable IFI (Aspergillus 22, Candida 4, Fusarium 1, Mucor 1) and 3 cases with possible IFI (all with lung localization). The sites of infection were: lung only 21/31 (69%), CNS 4/31 (12%), multiple sites 6/31 (19%); 13/31 (42%) cases occurred during pre-engraftment phase while 18/31 (58%) occurred after engraftment (with 12/18 cases after day 100). Advanced hematologic disease (relapsed or refractory) at time of transplant, history of pre-transplant IFI, presence of acute or chronic graft-versus-host-disease (GVHD), but not neutropenia, were significant risk factors (p<0,05). In the UR-BMT setting the incidence of IFI was significantly higher in patients who received a combination of immunosuppressive agents in the conditioning regimens (ATG ± Fludarabine ± Campath). Overall Survival after 100 days from diagnosis of IFI was only 20% and 22/31 (70%) deaths directly IFI related were reported. *Conclusions.* 1)IFI is still an important problem following R and UR-BMT and it is a significant cause of nonrelapse mortality. 2)Aspergillus sp. remain the most important aetiologic agent. 3)The incidence of IFI in UR-BMT is significantly higher than in R-BMT probably as a result of intensive immunosuppressive conditioning regimens in this setting. 3)IFI can develop late after engraftment (after day 100 from transplant) and without neutropenia. 4)Presence of GVHD, status of hematologic disease (relapsed/refractory) at transplant and history of pre-transplant IFI are important predisposing factors. 4)Retrospective studies, like this one, can be useful in order to identify high-risk BMT patients for which targeted and more effective strategies should be explored to prevent and treat IFI .

0381

INVASIVE FUNGAL INFECTIONS AFTER ALLOGENEIC STEM CELL TRANSPLANTATION: A SINGLE CENTER EXPERIENCE

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Background. From 1989 to 2004, 360 patients underwent allogeneic stem cell transplantation (ASCT) for malignant hematologic disease, using a conditioning regimen consisting of oral Busulfan 16mg/kg and iv Cyclophosphamide 60mg/kg (Bu4Cy2). 242 and 118 patients received grafts from family donors or matched unrelated donors (MUD), respectively. *Methods.* In this retrospective study we analyzed the incidence and outcome of invasive fungal infections (IFI). From 1999 oral Busulfan was targeted to a plasma level of 900ng/mL. Graft versus host disease (GvHD) prophylaxis was cyclosporine A (CiA) and a short course of iv Methotrexate. Treatment of established acute GvHD ≥ grade II, consisted of increased immunosupression with CiA and corticosteroids. Steroid refractory GvHD was treated with ATG or other immunosuppressants. The overall incidence of aGvHD was 46.9%; and aGvHD grade II-IV was 32.3%. The incidence of chronic GvHD was 35.6%; 22.8% limited and 12.8% extensive. Invasive fungal infections were defined according to EORTC guidelines. Only probable and proven IFI were registered. Routine primary antifungal prophylaxis was not given. *Results.* The incidence of IFI was 12.2% (n=44), 37 proven and 7 probable; Aspergillus species in 39%; Candida albicans in 39% and non-albicans strains in 16% of the infections registered. In two patients a definitive diagnosis could not be made. In one patient both Aspergillus and Candida species were isolated. Eleven patients had evidence of IFI before ASCT and secondary prophylaxis with Amphoterecin-B or Fluconazole was given. The temporal distribution differed between Candida and Aspergillus. Candida infections occurred at mean 66 days (range 9-255 post-Tx). Aspergillus infections at a mean of 146 days (range 32-519). In patients developing IFI before day 100, 63% had acute GvHD≥ grade II. Patients developing IFI after day 100 all had chronic GvHD with a risk of 6% and 16% in limited and extensive disease. The incidence of IFI was 9.2% and 18.6% in patients receiving grafts from family donor and MUD respectively. In logistic regression analysis the following were identified as factors predisposing to IFI: 1) graft from MUD, 2) corticosteroids; 3) acute GvHD. Stem cell source (bone marrow / peripheral blood stem cells) and disease were not found to be significant contributors. The mortality in patients with IFI was 71%, 77% and 88% in the non-albicans, albicans and aspergillus groups, respectively. 61% (N=27) and 4% (N=18) of patients had concomitant GvHD or CMV infection/disease. *Summary.* In this retrospective single center study of ASCT patients not receiving primary antifungal prophylaxis, documented IFI were found in 44 of 360 patients (12.2%), which is of the same order as in previous studies. The mortality was high; 71-77% among patients with Candida infections and 88% in patients with aspergillus infection. GvHD, corticosteroid therapy and graft from MUD were identified as risk factors.

0382

INTENSIFIED ENVIRONMENTAL SURVEILLANCE IN A STEM CELL TRANSPLANTATION WARD DURING CONSTRUCTION WORK

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Invasive aspergillosis is a serious complication with high mortality in allogeneic stem cell recipients and patients with acute leukaemia. Construction work close to a haematology ward is a known risk factor for aspergillus infections. At the Helsinki University Central Hospital, heavy construction work was performed from mid October until the end of year 2005 immediately adjacent to the 13-bed HEPA-filtered stem cell transplantation ward, located on the ground floor of the building. A protective barrier was built around three close-by ventilation ducts and around the construction area. The function of the air filters was followed by daily checking of the air pressure of ventilation channels. No increase in the pressure was seen. Regular surveillance sampling was performed in the ward. Particle counts were measured for particles above 0.3 microns in all patient rooms five times a week using Particle Scan Pro_ (IQ Air). The median particle count was 63-420 particles/litre. One peak of 1034 particles/litre was noticed. This was associated with heavy drilling during reconstruction work inside the hospital, four floors above the ward. The particle counts of the outside air at the hospital main entrance were significantly higher, between 110806 and 185645 spores/litre. Sampling for fungal spores was performed with a Surface Air Sampler, SAS 100 (pbi International, Italy). The samples were taken once a week from five different locations; three patient rooms, the construction area and the hospital main entrance. The samples from patient rooms were negative on 31 and positive on two occasions, one with Apergillus niger (1 CFU/m^3) and the other with nonpathogenic, environmental fungi. The samples from the construction area and the hospital main entrance were all positive, with 2-21 (median 9) CFU/m^3 and 1-31 (median 7) CFU/m^3, respectively. To rule out colonisation of the patient rooms and the patients, fungal cultures were performed. Surface samples from three different patient rooms were obtained once a week using contact plates. Of the 33 samples, 23 were negative and seven were positive but only for nonpathogenic fungi. Three samples were positive for aspergilli, two with Aspergillus fumigatus and one with Aspergillus versicolor. Swab samples were taken from both nares and the mouth of all patients and cultured for fungi on three occasions. All 70 nasal samples from 24 patients were negative. Of the 35 mouth samples, 18 were negative. Of the positive samples, 16 grew yeasts and one grew Aspergillus niger. This patient had been diagnosed with pulmonary aspergillosis prior to the beginning of the construction work. 55 patients were treated on the ward during this period. 15 allogeneic and 7 autologous stem cell transplantations were performed. Acute GVHD was treated in 11 patients. With a follow up time of 135 days from the beginning of the construction work, no aspergillus infections have been diagnosed in these patients. In conclusion, there were no indications of malfunction of the HEPA-filters. The protective measures seemed sufficient since no invasion of fungal spores to the ward was seen and the incidence of invasive aspergillus infections did not seem to increase.

0383

HICKMAN CATHETER-RELATED COMPLICATIONS IN ADULTS WITH HAEMATOLOGICAL/ONCOLOGICAL DISEASES: SINGLE CENTER EXPERIENCE OF 243 DEVICES

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Background. The use of tunnelled central venous catheters facilitates the management of haematological and oncological patients, but is not exempt from complications. *Aims.* We describe our experience with Hickman catheters in a tertiary care center in Spain between 1992 and 2005, trying to quantify the complications and characterize them. *Patients and Methods.* a retrospective analysis was performed on 243 consecutive double lumen Hickman-Broviac catheters (109 of fine diameter '9F- and 134 of gross diameter '13F-) inserted in 190 patients with haemopathies (131) or solid tumours (59). Catheters were inserted by experienced radiologist under fluoroscopic guidance. *Results.* Six early complications occurred, being the most relevant a subcutaneous tunnel necrosis. In 39,3% of the catheters a late complication was observed : 28,1% infectious, 9% mechanical, and 2% both of them. The infection location: 76.2% bloodstream, 13.8% exit site and 10% tunnel infection. The most common microorganisms isolated were coagulase-negative Staphylococcus (38.8%), Pseudomona sp. (13.8%), Klebsiella sp. (10%), Escherichia coli (8,8%) and other Gram negatives (20%). The main mechanical complications were: accidental removal (4%), device breaking and symptomatic thrombosis (1.6% in each case). The total catheter days at risk (CVC-days) were 20.902 (median: 54 days, range: 2-486 days). The overall complication rate was 4.9/1000 CVC-days (infectious rate 3.6/1000 CVC days: mechanical rate: 1.3/1000 CVC-days). The complication rate of gross catheters was 5.9/1000 CVC-days and 5.3/1000 CVC-days in the fine diameter devices. Complications were less likely to develop in catheters inserted in patients with haemopathy compared with those with solid tumours (3.8/1000 CVC-days vs 7.4/1000 CVC-days, p=0,002). *Conclusions.* in our experience, the Hickman catheter-related complication rate was 4.9/1000 CVC-days. Complications are more frequent in patients with solid tumours, but we did not found an statiscal significancy in the risk of complication related to the catheter diameter.

0384

ORAL VALGANCICLOVIR TREATMENT FOR CYTOMEGALOVIRUS DISEASE IN ALLOGENEIC STEM CELL TRANSPLANT RECIPIENTS

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*Background.*Valgancyclovir is a valyl ester of ganciclovir which is hydrolyzed to ganciclovir before reaching the systemic circulation, having higher bioavailability than oral ganciclovir. *Aims.* Despite the efficacy of valganciclovir has been demonstrated in immunosuppressed patients (CMV retinitis in AIDS patients, solid organ transplant recipients, patients treated with alemtuzumab), no data exist for the established dose and the indication of this formulation as CMV pre-emptive therapy in stem cell transplantation (SCT). *Methods.* Starting from July 2004, valganciclovir at a dose of 900 mg os once a day, was administered in 8 out-patients submitted to allogeneic SCT as pre-emptive therapy on the basis of detection of primary or reactivated CMV infection by positive antigenemia (Ag), or positive PCR. The median age was 43 years (range 21- 54 years). They were affected by acute myeloid leukemia (4), aplastic anemia (1), acute lymphoblastic leukemia (2) and low grade non-Hodgkin lymphoma (1). Six patients obtained complete remission after transplant. Acute GVHD occurred in 7 patients and chronic GVHD was noticed in 3 patients. Immunosuppressive regimen consisted of cyclosporine and methotrexate for all patients with addiction of steroids and mycophenolate or tacrolimus based on the development of acute or chronic GVHD. Antigenemia and PCR DNA for CMV were monitored twice a week. The median time of positivization of Ag and/or PCR for CMV was 45 days after transplant (range 35-63). Among evaluable patients, the mean baseline antigenemia level was 2/200.000 cells (range 1/200.000- 5/200.000), whereas the mean level of Dna viral copies was 6 × 103 /mL (range 0.320 × 103-24 × 103). *Results.* The negativization of PCR and/or Ag for CMV occurred in 6/8 patients (75%) at a median of 2 weeks from starting valganciclovir (range 2-3 weeks). One patient required further CMV treatment for a 2nd re-activation, at 252nd day, but obtained a rapid negativization after 1 week of therapy. Two patients, not achieving negativization, were shifted to foscarnet but developed CMV pulmonary disease and died at 189 days and 431 days. The cause of death was not attributable to CMV because they also developed recurrence of their malignancy. No significant increased myelo or nephro-toxicity was observed. *Conclusions.* Oral Valganciclovir was well tolerated showing efficacy and safety without significant hematological and/or extra-hematological toxicity; moreover it allowed good compliance and outpatient management.

0385

ANTIMICROBIAL PROPHYLAXIS AGAINST INFECTIOUS COMPLICATIONS OF AUTOLOGOUS STEM CELL TRANSPLANTATION: A RANDOMIZED PHASE II STUDY

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Background. The benefit of prophylactic antimicrobials in autologous stem cell transplantation (ASCT) remains to be answered. *Aims.* We performed a prospective randomized phase II comparison study to assess the benefit of prophylactic antimicrobials in ASCT. *Methods.* Forty consecutive patients with multiple myeloma (MM, 28 patients) or non-

Hodgkin's lymphoma (NHL, 12 patients) were stratified by disease and randomly allocated to receive (prophylaxis group, 21 patients) or not receive (control group, 19 patients) prophylactic antimicrobials just prior to administration of high-dose chemotherapy. Prophylactic antimicrobials consisted of ciprofloxacin (500 mg twice daily p.o.), fluconazole (100 mg twice daily p.o.) and acyclovir (400 mg every 8 h p.o.), starting 1 day before high-dose chemotherapy (high-dose melphalan for MM and BEAM for NHL) and continuing until absolute neutrophil count reached 500/mm^3 after nadir or infection occurred. Lenograstim 5 µg/kg/day was given from day 1 of ASCT. *Results.* At least one episode of fever occurred in 15/19 (79%) patients in the control group, compared with 12/21 (57%) patients in the prophylaxis group (p=NS). Microbiologically or clinically documented infections occurred in 4 patients (21%) in the control group, but none in the prophylaxis group (p=NS). Documented infections in the control group included 3 staphylococcal bacteremias and 1 herpes skin infection. No deaths, invasive fungal infections, or serious adverse events occurred in either group. The median duration of fever (9 days in the control group and 11 days in the prophylaxis group), therapeutic antimicrobial therapy (9 days in the control group and 11 days in the prophylaxis group), and hospital stay after ASCT (19 days in both groups) did not differ between the groups. Median time to neutrophil engraftment was 10 days in both groups and median time to platelet engraftment was 11 days in the control group and 12 days in the prophylaxis group. *Summary/Conclusions.* This small-sized prospective randomized phase II comparison showed no beneficial effect of antimicrobial prophylaxis in ASCT.

0386

PREDICTIVE FACTORS OF SEPTIC SHOCK AND MORTALITY IN NEUTROPENIC PATIENTS

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Neutropenia is the major risk factor for developing a serious infection. Bacteraemia still causes significant mortality (15-25%) among neutropenic patients with cancer. The purpose of this study was to identify risk factors for septic shock and for mortality in neutropenic patients with leukaemia and bacteraemia. Consecutive sample from 20 patients with acute myeloid leukaemia and bacteraemia was studied during 1 year (January 2003-Decembre 2003).All patients received empiric antibiotic therapies for febrile episodes with ceftazidime plus amikacin.110 neutropenic febrile episodes were noted: clinically documented 14.54%(16/110),microbiologically documented 16.36% (18/110),and fever of unknown origin 69.09% (76/110).Gram-negative organism caused 8 febrile episodes(8/18):Pseudomonas (5),klebsiella pneumoniae (3).Gram-positive organism caused 10 episodes: Staphylococcus (6),streptococci(2),enterococci(2).Pulmonary infection accounted for 25% of clinically documented infections.14 of the 110 febrile episodes were associated with septic shock causing mortality in 7 patients .The following variables influencing septic shock and mortality were analysed using logistic regression technique: site of infection, bacteria isolated, serum lactate, serum bicarbonate. In univariate analysis variables associated with septic shock were: pulmonary infection (OR=17, p=0.001),serum bicarbonate < 17 mmol/L (OR=68, p<0.001) and serum lactate > 3 mmol/L (OR=62, p<0.001).Variables associated with mortality: pulmonary infection (OR=83, p<0.001) and serum bicarbonate < 17 mmol/L (OR=61,p<0.001). In multivariate analysis two variables were associated with septic shock and mortality: pulmonary infection (OR=5, p=0.043) and serum lactate >3 mmol/L (OR=10, p=0.003). Elevated serum lactate (>3 mmol/L) and low serum bicarbonate (<17 mmol/L) at the onset of bacteraemia showed strong value in predicting septic shock and mortality in neutropenic patients.

0387

ANALYSIS OF COMPLICATIONS OF CENTRAL VENOUS CATHETERS IN ADULT HAEMATOLOGY PATIENTS.

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Background. Central venous catheters (CVC) are routinely employed in haematological patients, but their use may be complicated by many adverse events, such as catheter related infections (CR-I) and thrombosis (CR-T), that can induce an advanced removal. *Aim of the Work.* To analyse the fate of CVCs implanted from 2002 to 2004 in our haematological patients, with particular attention to: a) unfavourable events; b) relationship between surveillance culture and subsequent events. *Methods.* The records of patients whose CVC was implanted between January 2002 and December 2004 were reviewed retrospectively. All patients underwent high dose chemotherapy and/or stem cell transplantation. All patients received prophylaxis with ciprofloxacine or levofloxacine during aplasia periods. A protocol of periodic CVC surveillance cultures was followed. *Results.* A total of 210 CVC were considered. Distribution of patients for class of age was: 44 (21%) < 40 y; 78 (37%) from 40-60 y; 88 (42%) > 60 y. Diagnosis distribution was: acute leukaemia 98 (47%); lymphoma 75 (36%); multiple myeloma 30 (14%); others 7 (3%). 163 catheters (78%) were Groshong type. CVCs were inserted via subclavian vein in 198 cases (94%) and jugular vein in the remaining 12 cases (6%). Median CVC duration was 6 months, range 7 days to 18 months. Removal was independent from any CVC-related complication in 167 cases (80%), while it was performed in advance as a consequence of infections, malfunction and thrombosis in 25 (12%), 17 (8%) and 1 (<1%) respectively. At least one episode of subcutaneous flogistic event was evident in 118 cases (56%). Ninety nine of these subcutaneous infections (84%) were managed without CVC removal. Complete microbiological information on surveillance cultures were available in 157 cases, with one or more positive culture in 53 of them (34%). A subclavian thrombosis was demonstrated in 5 (2%) patients. Advanced CVC removal was statistically independent from age, diagnosis, subcutaneous infections and positive surveillance culture. No endocarditis was demonstrated. Among patients positive for surveillance cultures, Gram+ micro-organisms were more frequent then Gram- ones: 45 (85%) vs. 8 (15%). Cultures during a fever episode were performed in 145 cases with positive results in 77 (53%). Among patients with central positive cultures during fever episodes, Gram+ micro-organisms were isolated in 58 (75%), Gram- in 14 (18%), mycetes in 2 (3%) and both Gram+ and Gram- in two subsequent cultures in 3 (4%). A high concordance (89%) was evident among cultures of surveillance and those performed during fever episodes. Septic events were more frequent in patients with a positive surveillance culture than in those without (62% vs 34%, p<0.01). A similar predictive value was demonstrated by subcutaneous infections (48% vs 22%, p<0.01). *Conclusions.* Haematology patients frequently required an advanced CVC removal, mainly for infection complications. Both positive surveillance cultures and subcutaneous infections are highly predictive of subsequent septic events and can be useful to choose an empiric antibiotic therapy in case of fever.

0388

VARICELLA-ZOSTER VIRUS INFECTIONS AFTER HAEMATOPOIETIC STEM CELL TRANSPLANTATION: RELATIONSHIP WITH CD4+ CELL COUNTS.

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Introduction. Patients' undergoing haematopoietic stem-cell transplants (HSCT) are at high risk of varicella zoster virus (VZV) reactivation and antiviral prophylaxis only appears to delay events until cessation of prophylaxis. Their relationship with CD4 lymphocyte depletion has been extensively studied in HIV infection. This relationship is not well described in the oncology population, although several studies suggest that there is indeed increase risk for viral infections with low CD4 counts. *Patients and Methods.* Patients: 186 patients undergoing an auto and 26 an allo-HCST in our center from 1995 to 2005. The median age was 47 years (6-69). *Methods.* We report patients presenting VZV reactivation. Its clinical characteristics and CD4+ lymphocyte count were reviewed. *Results.* 25 patients (11.79%), 6 patients (23%) who had undergone allogeneic and 19 (10%) who had undergone autologous-HSCT, developed VZV reactivation a median of 147 days (range 23-636) post transplantation. 28% occurred in the first 3 months and 88% in the first year. In 3 patients reactivation occurred after the first year, all were allotransplant. Infection occurred in a localised dermatomal distribution in 92% of cases. 2 patients had disseminated cutaneous involvement. No patients had visceral dissemination or died. For patients (20%) had a CD4+ lymphocyte count of <200 cells/microL, thirteen (65%) between 200 and 400, and three patients (15%) >400. One patient with disseminated disease had a CD4+ lymphocyte count greater than 500 cells/microL. *Conclusion.* 1. VZV reactivation may be a significant infectious complication during HSCT recovery. Such infection is usually mild and localised. 2. Almost all patients had a CD4+ cell count <400/ microL. Our data indicated that it is not the only risk factor associated with reactivation. Immune system alterations in post transplant period are complex, and the role of monitoring lymphocyte subsets is uncertain. It neither seems to correlate with severity of disease. 3. We should investigate if these patients are candidates for vaccination because antiviral

prophylaxis only appears delay events as suggest the largest period of latency observed in allotransplant patients.

0389

AUDIT OF THE USE OF CT SCANNING AND RISK STRATIFICATION IN THE DIAGNOSIS OF INVASIVE FUNGAL INFECTIONS IN PATIENTS WITH HAEMATOLOGICAL MALIGNANCIES

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Background. Invasive fungal infections (IFIs) are a major cause of mortality and morbidity in neutropenic patients, but accurate early diagnosis remains difficult. Bronchoscopy is often problematic in such patients. As an alternative, computerised tomography (CT) examinations of the chest are non-invasive, readily available and show characteristic appearances if performed early in the course of the disease. The British Society for Medical Mycology (BSMM) has proposed standards for the diagnosis of patients with IFIs. *Aims.* We aimed to audit the radiological diagnosis of IFIs following these guidelines. In addition, we sought to assess the impact of risk stratification on the diagnosis of IFIs. Risk stratification models divide patients into low and high-risk groups. High-risk categories for invasive pulmonary aspergillosis are: - Neutropenia (neutrophils <= 0.2 $\times 10^9$/L.) due to intensive chemotherapy, if prolonged for > 21 days or concomitant steroid administration. -Post-allogeneic transplant if engraftment delayed or on steroids for graft versus host disease (GvHD). - Fungal spore exposure in neutropenia or recent invasive mould infection. *Methods.* 32 febrile, neutropenic patients initially treated with triple antibiotic therapy were enrolled in the study. CT chest studies were requested for the following indications: ·Fever unresponsive to 72 hours of antibiotics (18/39), or to 7 days of antibiotics in presence of another probable bacterial focus of infection (3/39) -Respiratory symptoms or signs (17/39, 8 with chest radiograph changes). -Positive fungal sputum cultures (1/39) CT was performed a median of 16 hours (range 1 hour - 45 hours) following request. A total of 39 examinations were performed in 32 patients. Patient details are outlined in Table 1. Statistical significance for a difference in the incidence of fungal infections between the low and high-risk groups was tested using the chi-square test. Results. CT diagnosis of pulmonary IFI was made in 11 patients (2 of whom had IFI with bacterial super-infection). Other diagnoses made were bacterial bronchopneumonia (9/39), atypical chest infection (1/39), GvHD (3/39), viral pneumonitis (2/39) and Pneumocystis carinii pneumonia (1/39). Bronchoscopy was performed in 4 cases, one of these was positive for Candida albicans. One patient died of IFI during the audit period. Patient risk was classified as high (HR: 27/39) or low (LR: 12/39) for invasive mould infections according to the Martino and Viscoli criteria. IFI was not confirmed in a single LR case, but was diagnosed in 11/27 HR patients (p=0.009). If CT imaging had been limited to high risk patients who met above criteria, a total of 12/39 examinations (31%) could have been avoided without missing a single case of IFI. *Conclusion.* Identification of patients at low risk of IFI by risk stratification may save resources, and reduce the use of empirical antifungal agents. CT is a useful diagnostic tool in IFIs.

Table 1. Patient characteristics (n=32)

Allograft recipients	5
Autograft recipients	9
Chemotherapy for NHL or CLL	4
Chemotherapy for AML	11
Chemotherapy for ALL	2
Chemotherapy for CML blast crisis	1

0390

CLINIC-ANALYTIC PROFILE AND USEFULNESS OF BONE MARROW SMEAR EXAMINATION AND SEROLOGICAL METHODS IN PATIENTS WITH VISCERAL LEISHMANIASIS EXPERIENCE OF OUR CENTER

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Background. The Mediterranean area is an endemic region of visceral leishmaniais (VL). With the advent of human immunodeficiency virus (HIV) infection, the number of cases of VL has dramatically increased in this area over the last years, mainly in adults. *Aims*. To analyze the clinic-analytic profile and usefulness of methods used in diagnosis of visceral leishmaniasis in our center. *Patients and Methods.* A total of 58 cases of VL were overviewed retrospectively from January 1989 to September 2005. Sex, age, clinic and analytic profile, diagnostic methods and HIV infection were studied. The median age was 29 years (range 8months-81 years) with 72% males (n=42) and 28% females (n=16). At diagnosis 97% presented fever (n=56), 91% splenomegaly (n=53), 71% hepatomegaly (n=53) and 19% pancytopenia. In 76% of patients (n=44) the hemoglobin was <100 gr/L, 34% (n=20) neutrophil count <1000 cells/mm^3 and 41% had thrombocytemia (<100×10^9/L). HIV infection affected 21 patients (36%) and the median hemoglobin of our series was 89 gr/L (range 53-135). The methods used in our center for the diagnosis of VL are bone marrow smear examination and serology methods (Indirect inmunofluorescent antibody test (IFAT) and ELISA). The statistical analysis was performed using the program SPSS v10.0. Results. The serodiagnosis of VL was positive in 28 cases and direct examination of the bone marrow smear yielded the diagnosis in 52 cases. The sensitivity of serologic studies was significantly lower in HIV(+) than in HIV^-patients (p=0,031). The 6 cases with negative examination of the bone marrow smear were HIV^- (p=0,057). *Conclusions.* The diagnosis of VL should be based in a direct examination of the bone marrow smear in combination with another diagnostic procedure. A negative serology is possible in HIV^+ patients. When PCR is not available the diagnosis method of choice in HIV^+/leishmania co-infected patients is direct examination of the bone marrow. In HIV(-) patients the sensitivity of serological methods is better than that of direct examination.

Thrombosis I

0391

DEEP VEIN THROMBOSIS AND PULMONARY EMBOLISM CAN BE TREATED AT HOME IN PATIENTS WITH CANCER

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Background. Outpatient treatment of deep vein thrombosis (DVT) has become a common practice in uncomplicated patients. Scanty data are presents in patients with comorbidity (such as cancer) or concomitant symptomatic pulmonary embolism (PE). Cancer patients with Venous Thromboembolism (VTE) are often excluded from home treatment because of high risk of bleeding and recurrent thrombosis. We tested the feasibility and safety of the home-treatment program in cancer patients with acute VTE. Material and *Methods.* Consecutive cancer patients having a confirmed episode of DVT or PE were treated as outpatients unless they required admission for other medical problems, were actively bleeding or had pain treated with i.v. narcotics. As anticoagulants, patients received standard therapy with Low Molecular Weight Heparin (LMWH) followed by warfarin or LMWH alone, at therapeutic dosages; all of them were treated for 6 months. At the index visit, an educational program for self-injection and clinical surveillance was implemented. *Results.* Over a period of 3 years, 207 patients with cancer and acute VTE (139 with DVT and 68 with PE) were evaluated; 36 (17.4%) of them had metastatic disease. Treatment with standard anticoagulation (LMWH followed by warfarin) was given to 106 (51.2%) while LMWH alone to 102 (48.8%) patients. One hundred and twenty-seven patients (61.3%) (91 with DVT and 36 with PE) were entirely treated at home. In the remaining patients, reasons for hospital admission (n. 80) were poor compliance [22, (27.5%)], concomitant serious illness [52 (65%)] and refusal of home-treatment [6 (7.5%)]. There were no differences between patients treated at home and those hospitalized with regard to gender, mean age, site of cancer, presence of metastases and choice of anticoagulants (Table).

	Standard in hospital		Home Therapy		P value
Number of patients	48 DVT	32 PE	91 DVT	36 PE	n.s
Mean age (range)	68.6 (37-92)		61.5 (32-90)		n.s
Males	43 (56.2%)		67 (52.7%)		n.s
Proximal DVT	43 (89.5%)	6 (18.7%)	82 (90.1%)	7 (19.4%)	n.s
Distal isolated DVT	5 (10.4%)	2 (6.2%)	9 (9.8%)	2 (5.5%)	n.s
Symptoms of PE*	7 (12.5%)	---	8 (8.7%)	---	n.s
Metastatic cancer	10 (20.8%)	7 (21.8%)	13 (14.2%)	6 (16.6%)	n.s
Site of cancer, n (%): Gastrointestinal Genitourinary Breast Lung Haematologic	33 (41.2) 21 (26.2) 26 (32.5) 6 (7.5) 4 (5)		56 (44.1) 26 (20.4) 24 (18.9) 14 (11) 7 (5.5)		n.s.
Mean time from cancer to VTE diagnosis	30.8 months		28.9 months		n.s.
Ongoing chemo-, or radio or hormone therapy, n (%)	32 (40)		43 (33.8)		n.s.
Co-morbidity	21 (43.7%)	21 (65.6%)	55 (60.4%)	20 (55.5%)	n.s
In-hospital stay	8±2 days		3.1 hours		< 0.0004

* In DVT patients only

Table 1. Clinical events at 6 months of follow-up.

After 6 months, recurrent DVT, PE and major bleeding occurred in 6.5%, 5.5.% and 1.5% of patients treated at home, and 8.3%, 9.3% and 2% of those hospitalised. These differences were not statistically significant (p=0.58). Twenty-seven patients (33%) in the hospitalized group and 33 (26%) in the home-treatment group died as a consequence of neoplasm. *Conclusions.* These results indicate that, regarding cancer patients with acute DVT and/or PE, there is no difference between hospitalised and home-treated patients in terms of major outcomes.

0392

A GLOBAL ASSAY FOR THE ASSESSMENT OF LOW MOLECULAR WEIGHT HEPARINS ANTITHROMBOTIC ACTIVITY *IN VITRO*

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Background. Low molecular weight heparins (LMWHs) are derived from unfractioned heparin (UFH) by depolymerization. Thus, they present biochemical and pharmacological differences and the ratio of the anti-Xa/anti-IIa activities varies from one product to another. LMWHs have no effect on prothrombin time and there is no a global clotting assay for the *in vitro* assessment of their antithrombotic activity. Furthermore, the anti-Xa activity measurement, which is routinely used in clinical practice for monitoring the anticoagulant treatment with LMWHs, has a limited predictive value concerning the clinical outcome (thrombosis or bleeding). *Aims.* The aim of the present study was to assess the LMWHs global antithrombotic activity by using a rather physiologically relevant system. For this purpose we used the Thrombogram-Thrombinoscope assay, a dynamic assay which describes all the phases of thrombin generation (TG) process (initiation, amplification and inhibition of TG as well as the integral amount of generated thrombin). *Methods.* TG was assessed after tissue factor (TF) pathway activation in platelet rich plasma (PRP) (1.5×105 platelets/μL) using diluted thromboplastin (Dade Innovin®, 1:1000 final dilution). We studied five different LMWHs (bemiparin, enoxaparin, nadroparin, dalteparin and tinzaparin), as well as UFH at five different prophylactic and therapeutic anti-Xa final concentrations. These agents were added to control plasma from 14 healthy volunteers with equivalent anti-Xa concentrations. TG was initiated by adding the triggering solution containing CaCl2 and the fluorogenic substrate. The analyzed TG parameters are the lag-time, the maximal concentration of thrombin (Cmax), the time to reach Cmax (Tmax), the TG velocity and the endogenous thrombin potential (ETP). *Results.* Bemiparin had almost no effect on TG, with concentrations below 0.60 anti-Xa IU/ml. Enoxaparin, nadroparin and dalteparin showed a similar potency in inhibiting TG at equal anti-Xa concentrations. Tinzaparin proved to be the most active LMWH in inhibiting TG and had a similar potency to UFH. Tinzaparin and UFH, with the lowest anti-Xa/anti-IIa ratio, exerted their inhibitory effect mostly by prolonging lag-time and Tmax and by reducing TG velocity, especially at concentrations below 0.40 anti-Xa IU/mL. Besides, UFH totally inhibited TG, as expressed by ETP, at a concentration over 0.40 anti-Xa IU/mL. For a given anti-Xa/anti-IIa ratio characterizing each LMWH the IC50 for each parameter was different. The IC50 for the reduction of the velocity of TG was lower as compared to the IC50 for the other parameters. (Table 1).

Table 1. The IC50% for each parameter of TG.

	Lag-time (50)↑	*Tmax (50)*↑	*ETP (50)*↓	*Cmax (50)*↓	*Velocity (50)*↓
Bemiparin	>1IU/mL	>1IU/mL	0.98IU/mL	0.85>IU/mL	0.68>IU/mL
Enoxaparin	0.62IU/mL	0.58IU/mL	0.55IU/mL	0.45>IU/mL	0.38>IU/mL
Nadroparin	0.80IU/mL	0.75IU/mL	0.55IU/mL	0.45>IU/mL	0.38>IU/mL
Dalteparin	0.65IU/mL	0.65IU/mL	0.50IU/mL	0.42>IU/mL	0.38>IU/mL
Tinzaparin	0.35IU/mL	0.28IU/mL	0.35IU/mL	0.25>IU/mL	0.18>IU/mL
UFH	0.05IU/mL	0.10IU/mL	0.30IU/mL	0.25>IU/mL	0.18>IU/mL

Summary/Conclusions. Our study reinforces the concept of LMWH heterogeneity and the important effect exerted by the additional anti-IIa activity of LMWHs, combined with their anti-Xa activity. Thus, their characterization can be made through their ability to inhibit TG and not only their anti-Xa/anti-IIa ratio. Furthermore, the anti-IIa inhibitory activity of heparins is primarily expressed by prolonging the lag-time and the Tmax and by reducing the TG velocity. The clinical relevance of our findings has to be studied, while the use of TG assay should be considered as a potent method to monitor anticoagulant treatment with LMWHs in the routine hematological laboratory.

0393

RISK OF RECURRENT VENOUS THROMBOEMBOLISM ASSOCIATED WITH PREGNANCY IN WOMEN WITH A HISTORY OF VENOUS THROMBOSIS

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Background. Previous estimates of the rate of recurrent venous throm-

boembolism (VTE) during pregnancy in women with a history of VTE have vary between 0 and 13%. Therefore, the decision to administer or withhold heparin especially in the antepartum period has been discussed controversial. In a recent study by Brill-Edwards *et al.* (N Engl J Med 2000;343:1439-44), no recurrences of VTE occurred in women (n=44) who had a previous episode of thrombosis that was associated with a temporary risk factor and who also had no evidence of thrombophilia. Based on these results, antepartum heparin prophylaxis is not routinely recommended in women without thrombophilia whose previous episode of thrombosis was associated with a temporary risk factor (ACCP guidelines 2004). The aim of our study was to evaluate the risk of recurrent pregnancy-associated thrombosis in women with a history of VTE. Materials and *Methods.* We retrospectively studied 198 women with at least one pregnancy (275 pregnancies in total) after a one previous episode of VTE. Sixty-three women (81 pregnancies) were excluded from the analysis because of antepartum heparin prophylaxis. *Results.* In the subgroup of women without heparin prophylaxis (n=135), 15 (7.7%) thromboembolic events occurred antepartum in 194 pregnancies. Further subgroup analysis, stratified for the nature of first VTE, gave the following number of antepartum VTE per number of pregnancies: 2 VTE/19 pregnancies (10.5%) in 14 women (first VTE: immobilization), 4 VTE/33 pregnancies (12.1%) in 24 women (first VTE: surgery), 5 VTE/69 pregnancies (7.2%) in 46 women (first VTE: oral contraception), 2 VTE/58 pregnancies (3.4%) in 40 women (first VTE: pregnancy), 2 VTE/15 pregnancies (13%) in 11 women (first VTE: idiopathic). Nine of the 15 women with VTE (7/13 women with first VTE triggered by temporary risk factor; 2/2 women with first idiopathic VTE) had a heterozygous factor V Leiden G1691A or prothrombin G20210A gene mutation. In the postpartum period, 16 VTE in 194 pregnancies occurred after live birth in the 135 women without heparin prophylaxis. Nine of these 16 women had a heterozygous FVL or prothrombin G20210A gene mutation. In Conclusion, the risk of recurrent antepartum VTE was similar in women with and without factor V Leiden G1691A or the prothrombin G20210A gene mutation and did not differ between women with first VTE triggered by a transient risk factor or an idiopathic first VTE. In addition to recommended postpartum heparin prophylaxis, our data support the need for a routine antepartum prophylaxis in women without thrombophilia whose previous episode of thrombosis was associated with a temporary risk factor.

0394

MAJOR HAEMORRHAGE BEFORE VENOUS THROMBOEMBOLISM: DIFFERENT OUTCOMES DEPENDING ON THE BLEEDING SITE

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Background. Patients with a recent episode of major bleeding are usually excluded from clinical trials. The management of these patients is not evidence based and their outcomes are unknown. *Aims.* To study outcomes of patients with VTE and a recent episode (< 30 days) of major bleeding before VTE diagnosis, according to the bleeding site and the time interval between bleeding and VTE. *Methods.* Analysis of the data from a prospective, multicentre registry of VTE (RIETE) entering consecutive patients with VTE diagnosed by objective tests. Patient characteristics, antithrombotic treatments and 3-month outcomes were recorded. *Results.* Of the 12.302 patients enrolled up to July 2005, 306 (2,5%) patients had had a recent episode of major bleeding, 106 (38%) gastrointestinal (GI), 94 (30%) intracraneal, and 96 (32%) from other sites. When compared with the group of patients without recent haemorrhage, the mortality rate (14,1% vs. 8,0%), major haemorrhage rate (6,2% vs. 2,3%) and fatal haemorrhage rate (2,6% vs. 0,5%) were significantly higher (p<0,01) in the recent bleeding group. With the exception of the intracraneal site, previous bleeding patients had an increased risk of new bleeding (*GI* HR 3,1; 95% IC: 1,9-4,9. *Other* HR 3,0; 95% CI:1,6-5,4) and death (*GI* HR 1,9; 95% IC: 1,2-3,1. *Other* HR 2,0; 95% CI:1,2-3,3). Episodes of major bleeding were associated with previous GI haemorrhage (HR 2,8; 95% IC 1,4-5,3). A time interval of less than 2 weeks between major bleeding and VTE diagnosis was also associated with an increased risk of any episodes of bleeding (HR 4,4; 95% IC: 2,4-8,), major bleeding (HR 2,4; 95%IC 1,2-5,0) and death (HR 2,7; 95% IC: 1,7-4,1). *Conclusion.* The risk of new bleeding episodes or death during a 3-month follow-up was increased in patients with VTE and 1) previous bleeding other than intracranial and 2) bleeding episodes occurring less than two weeks before VTE diagnosis. The antecedent of recent intracraneal bleeding identifies a subgroup of patients with VTE and a better prognosis.

0395

SUBOPTIMAL DOSES OF LOW MOLECULAR WEIGHT HEPARIN IN THE TREATMENT OF VENOUS THROMBOEMBOLIC DISEASE

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Background. A number of patients with venous thromboembolism (VTE) are treated with suboptimal doses of low molecular weight heparin (LMWH). However, there are no clinical trials that have established the efficacy of these doses. *Aims.* The objective of this study was to evaluate the evolution of patients treated with suboptimal LMWH (60-149 UI/Kg/d) as compared with patients treated with standard doses (150 UI/Kg/d). *Methods.* Analysis of data from a prospective, multicentre registry of VTE (RIETE) entering consecutive patients with VTE diagnosed by objective tests. Patient characteristics, antithrombotic treatments and 3-month outcomes were recorded. *Results.* Up to July 2005, 10.524 were diagnosed with deep vein thrombosis (DVT) or pulmonary embolism (PE) (n=4.321) and treated initially with LMWH; 1.547 (14.7%) patients received suboptimal LMWH (mean, 122 UI/Kg/d) and 8.977 (85,3%) patients received full-dose LMWH (mean, 191 UI/Kg/d). Suboptimal doses of LMWH were significantly (p<0.05) associated with younger patients (63.3 vs. 65.7 years), outpatient treatment (26.7% vs. 12.5%), use of vena cava filters (2.4% vs. 1.2%), recent episodes of major bleeding (4.3% vs. 1.6%) and renal failure (15.6% vs. 13%). Standard doses of LMWH were more frequently used in patients with proximal DVT (83,4% vs. 80,6%), and PE (42,5% vs. 28.8%). At the end of the follow-up, there were no significant differences in the rates of mortality (7.7% vs. 7.8%), VTE recurrence (2.7% vs. 2.3%), or fatal haemorrhage (3.2% vs. 2.6%) between the suboptimal and the standard group. Neither were there any differences in the subgroup of patients with PE (mortality, 10.1% vs. 9.7%; recurrence, 2.7% vs. 2.5%; fatal haemorrhage, 0.9% vs. 0.6%) nor in the whole group after a 2-week follow-up (mortality, 2.7% vs. 2.9%; recurrence, 0.8% vs. 0.8%; fatal haemorrhage, 0.3% vs. 0.3%). During the first two weeks, major bleeding rate was significantly higher in the suboptimal LMWH group (2.0 vs. 1.1%; p=0.003). In multivariate models, entering relevant baseline risk factors, neither mortality nor recurrence nor bleeding was associated with the use of suboptimal doses of LMWH. *Conclusion.* Suboptimal doses of LMWH were more frequently given to patients with prior recent bleeding, renal failure and less critical clinic manifestations, and were not associated with an increased mortality or recurrence rate.

0396

ELEVATED PROTHROMBIN FRAGMENT F1+2 LEVELS DURING PREGNANCY IN WOMEN WITH PREVIOUS VENOUS THROMBOEMBOLISM

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Background. Changes in blood coagulation and fibrinolysis during pregnancy create a state of hypercoagulability. This phenomenon predisposes to venous thromboembolism. Women with prior venous thromboembolism are believed to have a higher risk of venous thromboembolism in a subsequent pregnancy. The risk is higher if the past episode was unprovoked, and the risk is higher if the past episode was associated with biochemical abnormalities such as factor V Leiden G1691A (factor V Leiden). Since the positive predictive value of factor V Leiden and other risk determinants for a pregnancy associated thrombosis is low, additional indicators of hypercoagulability are needed. Indicators of hypercoagulation in normal pregnancy are increased levels of prothrombin fragment 1+2. *Aim.* We hypothesized that women with factor V Leiden or a previous venous thromboembolism are at a higher hypercoagulable state during subsequent pregnancies than women without prior thrombotic complications or without factor V Leiden. *Patients and Methods.* In a prospective study, we determined prothrombin fragment F1+2

over pregnancy among 109 women (175 measurements) with previous venous thromboembolism, and among 75 pregnant women (75 measurements) without previous venous thromboembolism. The prothrombin fragment F1+2 levels were statistically analyzed over time using a mixed model. This model allows a longitudinal analysis of the influence of a between-subjects factor (e.g. history of thrombosis) on prothrombin fragment F1+2 levels, the influence of a within-subjects factor (weeks of gestation) on prothrombin fragment F1+2 levels, and the interaction of the history of thrombosis and weeks of gestation representing a change of risk factor-dependent differences over time (weeks of gestation). *Results.* Among women with a previous history of venous thrombosis, prothrombin fragment F1+2 values were significantly higher during the course of pregnancy than among pregnant women without venous thromboembolism (p=0.001). The results were adjusted for the physiological increase of prothrombin fragment F1+2 over pregnancy and independent from heparin prophylaxis. In addition, factor V Leiden was independently associated with increased levels of prothrombin fragment F1+2 (p=0.03). *Conclusion.* Thus, determination of indicators of hypercoagulation like prothrombin fragment F1+2 represent an additional approach independent from known and unknown risk determinants of thrombosis to identify women at risk for venous thromboembolism during pregnancy.

0397

RECURRENT FETAL LOSS: PROSPECTIVE EVALUATION OF THE EFFICACY OF THREE DIFFERENT THROMBOPROPHYLAXIS REGIMENS: ASPIRIN VERSUS LOW MOLECULAR WEIGHT HEPARIN VERSUS LOW MOLECULAR WEIGHT HEPARIN PLUS ASPIRIN

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Background. Growing evidence suggests that thrombophilia is associated with an adverse pregnancy outcome, but there is a lack of controlled trials of antithrombotic prophylaxis to prevent pregnancy complications. *Aims.* The aim of our study was the prospective evaluation of the efficacy of three different thromboprophylaxis regimens in women with two or more unexpected pregnancy loss. *Methods.* A total of 361 women with pregnancy loss were studied for thrombophilia: 226 (72.6%) did not present thrombophilia; 99 (27.4%) were positive for one or more thrombophilic parameters. 94/361 women got pregnant (none of these patients presented APA syndrome): 56 (59.6%) with negative congenital thrombophilic screening, 38 (40.4%) with positive congenital thrombophilic screening. These 94 patients were randomly assigned to one of the three thromboprophylaxis regimens from the 8th week of pregnancy: low dose aspirin 100 mg daily (arm A), enoxaparin 40 mg daily (arm B), aspirin 100 mg plus enoxaparin 40 mg daily (arm C). All patients with thrombophilia were treated with implemanted enoxaparin between the 36th week of pregnancy and the 6th week after delivery. 4. *Results.* Thromboprophylaxis therapy was associated with 73 (77.7%) live births and 21 (23.3%) pregnancy losses: in a total of 305 previous pregnancies, we observed 37/305 (12.1%) live births and 268/305 (87.9%) pregnancy loss (p<0.0001)). In the 56 patients with negative thrombophilia screening, thromboprophylaxis was associated with 49 (87.5%) live births and 7 (12.5%) pregnancy losses: in a total of 150 previous pregnancies, these negative patients had 18/150 (12%) live births and 132/150 (88%) pregnancy losses (p<0.0001). Considering the three different therapeutic regimens, we noted in arm A: 14/19 (73.7%) live births and 5/19 (26.3%) pregnancy losses; arm B: 16/18 (88.9%) live births and 2/18 (11.1%) pregnancy losses; arm C: 19/19 (100%) live births. In the 38 patients with positive thrombophilia screening, the thromboprophylaxis was associated with 24 (63.2%) live births and 14 (36.8%) pregnancy losses: in a total of 155 pregnancies, these positive patients had 19/155 (12.2%) live births and 136/155 (87.8%) pregnancy losses (p<0.0001). In these patients, considering the three different therapeutic regimens, we noted in arm A: 3/12 (25%) live births, and 9/12 (75%) pregnancy losses; in arm B: 10/13 (76.9%) live births and 3/13 (23.1%) pregnancy losses; in arm C: 11/13 (84.6%) live births and 2/13 (15.4%) pregnancy losses. *Conclusions*. Our study shows that thromboprophylaxis therapy is effective in women with recurrent pregnancy losses. In the negative thrombophilic patients, thromboprophylaxis is effective: (87.5%) live births. In these patients, no difference was found in the three therapeutic regimens. In the positive thrombophilic patients, therapy with enoxaparin or aspirin plus enoxaparin was more effective than aspirin treatment (p=0.0169 and p=0.0048, respectively). No difference was found between enoxaparin versus aspirin plus enoxaparin.

0398

THE RISK OF RECURRENT VENOUS THROMBOEMBOLISM IN PREGNANCY AND PUERPERIUM WITH NO ANTITHROMBOTIC PROPHYLAXIS

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Background. Whether or not pregnant women with a previous venous thromboembolism (VTE) should receive antithrombotic prophylaxis is a matter of debate. *Aims.* To estimate the probability of recurrent VTE during pregnancy and puerperium in women stratified according to the presence of inherited thrombophilia and/or the circumstances of the first VTE. *Methods.* We studied a retrospective cohort of 1401 women with a first VTE occurred before 40 years of age and referred to our centers for laboratory evaluation. The previous clinical history was taken at the admission blinded to the laboratory results. All the events were objectively diagnosed. Inherited thrombophilia was defined as the presence of deficiency of antithrombin, protein C, and protein S, factor V Leiden, prothrombin G20210A. Women with antiphospholipid antibodies were preliminarly excluded from the study. After the first VTE 197 women were pregnant at least once. Further exclusion criteria of women were a history of recurrence between the first thrombosis and pregnancy (n=12), antithrombortic prophylaxis during all the pregnancies after the first VTE (n=44), and no pregnancy ended with live birth (n=7); thus 134 of them (48 with thrombophilia) had after a single VTE at least one pregnancy without obstetric complications in the absence of antithrombotic prophylaxis and completed 202 pregnancies. *Results.* We recorded 12 antepartum and 22 postpartum first recurrent VTE events, for a probability of recurrence of 6% (95% CI 3.5-10.3) and 11.8% (95% CI 7.9-17.2), respectively. In the antepartum period the risk was 9.2% (95% CI 4.0-19.9) in women with a first unprovoked VTE and 8.1% (95% CI 4.0-15.8) in those with a first pregnancy-related VTE, whereas no recurrence was recorded in women with a first VTE provoked by other risk factors. In the puerperium the risk was 19.7% (95% CI 12.5-29.7) in women with a first pregnancy-related VTE; the risk was 1.9% (95% CI 0.3-1.0) in women with a first unprovoked VTE, 8.8% (95% CI 3.0-22.9) in women with a surgery-related first VTE, and 10% (95% CI 2.7-30.1) in women with a first VTE during oral contraceptives.Women with a first pregnancy-related VTE had a 3.3-fold (95% CI 1.4-7.9) risk of postpartum recurrence than women with a first VTE occurred in other circumstances. Carriers of factor V Leiden had a probability of postpartum recurrence of 26.4% (95% CI 14.6-43.1), which is 2.8-fold (95% CI 1.3-6.0) increased in comparison to women without thrombophilia. In women with both factor V Leiden and a first pregnancy-related VTE the risk of postpartum recurrent VTE was 8.4-times higher than in non-carriers with other circumstances of first VTE (95% CI 2.5-28.3). *Conclusions.* The probability of recurrent VTE among pregnant women with previous VTE is not negligible, particularly in the puerperium, in those with a first pregnancy-related VTE, and in carriers of factor V Leiden. Other than in the puerperium, antithrombotic prophylaxis may be warranted antepartum in women with a first unprovoked or pregnancy-related VTE.

0399

A THROMBOELASTOGRAPHIC STUDY IN WHOLE BLOOD EMPLOYING A FIBRIN POLYMERIZATION INHIBITOR (PEFABLOC?) AND AN INHIBITOR OF ACTIN POLYMERIZATION (CYTOCHALASIN D)

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Background. Minimal tissue factor (TF) triggered whole blood thromboelastography (TEG) provides a valuable tool for studying the kinetics of thrombus formation (expressed by the parameters R, k and λ-angle) and the physical characteristics of the thrombus, such as its firmness and the elastic modulus shear expressed by the parameters of maximal amplitude (MA) and the G respectively. *Aims.* We studied the influence of fibrin polymerization and platelet functional status on the thromboelastographic trace after minimal TF pathway activation in whole blood using increasing concentrations of a fibrin polymerization inhibitor (Gly-Pro-Arg-Pro-OH.AcOH; Pefabloc-FG) and an inhibitor of actin polymerization (Cytochalasin D). *Methods.* Coagulation was triggered in a plastic disposable cup containing 20 µl CaCl2 (0.2M) and 10 µl of diluted thromboplastin by the addition of 330 µl whole blood, supplemented with Pefabloc-FG_or Cytochalasin D. Data acquisition was done during

60 minutes, and five coagulation parameters were analysed: (1) R-time (min): time from the start of the sample run to the point of first significant clot appearance corresponding to an amplitude of 2 mm, (2) k-time (min): time from R-time until the level of clot firmness reaches an arbitrary value of 20 mm, (3) α angle (degree): reflects the kinetics of clot development, (4) MA (mm): maximum amplitude reflects the maximum strength of the developed clot, (5) G (dyn/cm^2): reflects clot firmness. *Results.* Pefabloc-FG at concentrations higher than 5 mg/mL prolonged the R and k-times and decreased the α angle in a concentration-dependent manner but it did not modify MA and G. Pefabloc-FG at 5 mg/mL, completely inhibited thrombus formation. Cytochalasin D did not modify R-time but decreased the α-angle, MA and G. The effect of cytochalasin D was pronounced on MA and G. A combination of Pefabloc-FG (0.5 mg/mL) and cytochalasin D (50 μM) significantly decreased α-angle compared to control as well as their single effect. However, G was dramatically reduced in the presence of cytochalasin D, without any additional effect by Pefabloc-FG. *Conclusions.* This study confirms the importance of fibrin polymerization on the kinetics of thrombus formation and demonstrates the close association between the quality of the thrombus and the functional status of platelets. Normal platelet contractile forces are of major importance for the maximum amplitude of TEG which is related to the strength and elastic modulus of the thrombus.

0400

PATIENTS WITH ANTIPHOSPHOLIPID ANTIBODIES HAVE A HIGH INCIDENCE OF ANTI-ADAMTS13

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Background. Thrombotic thrombocytopenic purpura (TTP) and antiphospholipid syndrome (APS) are autoimmune diseases associated with thrombosis. TTP is associated with thrombocytopenia, microangiopathy, variable multi-organ ischaemia and reduced ADAMTS 13 activity. The mechanism of thrombosis in APS is unclear, but catastrophic cases of APS can result in similar microangiopathic features to those of TTP. *Aim.* Autoantibodies that neutralise ADAMTS13 are commonly found in patients with acquired idiopathicTTP, but their incidence in other thrombotic autoimmune diseases is not well investigated. Method: In our ongoing study we have currently assessed 76 patients with antiphospholipid antibodies (66/76 primary APS, 8/76 SLE, 2/76 other secondary APS). *Results.* We found IgG ADAMTS13 antibodies (Imubind ELISA Kit, American Diagnostica Inc) in 39/76 (51%). The ELISA normal reference range was <9.6, and these patients had a mean of 15.3 micrograms per millilitre (range 9.7-53.5). Of 43 patients assessed for ADAMTS activity, 12 (27.9%) had reduced ADAMTS13 activity by collagen binding technique (median 18%, range 0-48.5%, NR 66-126%). 7 patients were positive for IgG anti-ADAMTS13 and had low activity, however, some patients were only abnormal in one or the other assay. Low ADAMTS13 activity was not associated with excessively high VWF antigen suggesting that ADAMTS13 was not depleted due to high VWF turnover. Antibodies to ADAMTS13 in TTP are primarily inhibitory. In the APS population, it is not known whether ADAMTS13 IgG antibodies neutralise activity or cause immune complex formation with subsequent removal. In addition, some patients may have other classes of antibody to ADAMTS13 (eg. IgM or IgA). We intend to further characterise these antibodies. *Conclusion.* We hypothesize that the presence of ADAMTS13 antibodies in APS may contribute to the pathophysiology of thrombosis in APS.

0401

B-VITAMIN SUPPLEMENTATION INCREASES MARKERS OF ENDOTHELIAL FUNCTION IN PATIENTS WITH VENOUS THROMBOEMBOLISM

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Background. Mild hyperhomocysteinemia is associated with an increased risk of venous thromboembolism (VTE) and other cardiovascular diseases. The pathophysiological mechanism that explains this association is unclear, but *in vitro* studies suggest impaired endothelial function in hyperhomocysteinemic patients. Whether decreasing homocysteine with B-vitamin supplementation interferes with its effects on the endothelium is still to be determined. *Aims.* This study was designed in order to evaluate the correlation between homocysteine and markers of endothelial function and to evaluate the effect of vitamin supplementation on these markers in patients with VTE. *Methods.* This study was a multicentre, randomized, double-blind, placebo-controlled trial. We randomized 105 patients with a first event of objectively confirmed VTE, aged between 18 and 70 years, to receive either vitamin supplementation (folic acid 5 mg, vitamin B6 50 mg and vitamin B12 0.4 mg) or placebo. Blood was collected at randomization and 8 weeks after the intervention period. *Results.* Ninety-nine (94.3%) completed the 8-week period of treatment. We compared patients with basal homocysteine above the highest tertile (12.6 micromol/L) with those below the lowest tertile (9.9 micromol/L). There was no difference in the levels of t-PA, plasminogen activator inhibitor type1 (PAI-1), Factor VIII:C and von Willebrand factor antigen (vWF) between the two groups. Vitamin supplementation decreased the homocysteine median levels from 10.7 to 8.1 micromol/L (29% reduction). There was a significant increase in the levels of tissue plasminogen activator (t-PA) from 6.1 to 9.0 nmol/L in the group treated with vitamins, (p=0.0008, Wilcoxon rank-sum test) and also in the group treated with placebo, although less evident (from 8.0 to 9.5 nmol/L, p=0.03). PAI-1 levels did not change after 8 weeks both in the vitamin and in the placebo groups. Both t-PA and PAI-1 levels significantly increased only in the group of patients above the highest tertile of homocysteine who received vitamin supplementation (p=0.0004 and p=0.014, respectively). There was no change in the levels of these two markers in patients with homocysteine levels below the lowest tertile or in patients who received placebo with higher and lower homocysteine levels. vWF and factor VIII:C were unaffected by both vitamins and placebo, even in patients above the highest tertile of homocysteine. *Conclusions.* In patients with VTE, homocysteine reduction by B-vitamin supplementation caused a significant increase on t-PA. In patients with higher levels of homocysteine both t-PA and PAI-1 were increased by vitamins, although the basal levels of both markers were similar to the patients with lower levels.

0402

D-DIMER LEVEL IS ASSOCIATED WITH THE SEVERITY OF PULMONARY EMBOLISM

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Background. PE is a potentially fatal condition with a 3-month mortality rate reaching 15%. D-dimer is widely used as an initial test in the management of suspected PE. Risk factors like hypotension, right ventricular dysfunction and elevated cardiac biomarkers are associated with adverse prognosis and poor outcome. Patients carrying such risk factors may require more intensive treatment and usually benefit from thrombolysis. *Aim.* To investigate the association between the level of D-dimer and the severity of PE as determined by clinical, biochemical and radiological parameters. *Patients and Methods.* From Feb 2002 to Dec 2003, 99 consecutive patients were diagnosed with PE using 4-detector row CT at the Østfold Hospital Trust Fredrikstad, Norway. All patients had elevated STA-Liatest D-dimer (cut-off ≤0.4 mg/L). Pulmonary artery obstruction index (PAOI) and Right Ventricular/Left Ventricular ratio (RV/LV) were assessed retrospectively. Troponin T (TNT) was assayed 67 patients within 48 hour following the establishment of diagnosis; levels >0.01 ng/mL were regarded as indicating myocardial injury. *Results.* The median value for D-dimer was 5.0 mg/L (inter-quartile range: 1.8,12.2). There was a significant linear association (p<0.0001) between log-D-dimer, and between log-RV/LV (r=0.45), log-PAOI (r=0.5), PaO2 (r=0.40), P(A-a)O2 gradient (r=0.45) and log-duration of hospital stay (r=0.25). The multivariate analysis showed an increased association between Log-D-dimer and between Log-RV/LV ratio (r= 0.54, p<0.0005) and log-PAOI (r=0.52, p<0.0005) after adjusting for age, gender and for the duration of symptoms. A significant association was found between D-dimer and the most proximal level of PE. Moreover, a significant dose-response relationship was found between the level of D-dimer (low level = lower quartile of D-dimer values, n=26; intermediate level = interquartile range, n=48; high level = upper quartile, n=25) and between TNT (table) and the frequency of thrombolysis. Of the 96 patients who received treatment (PE was overlooked in 3 patients), 84 patients (87%) were treated with heparin, while 12 patients (13%) received systemic thrombolysis. In the subgroup of patients with D-Dimer in the upper quartile, 8 patients (33%) received thrombolysis, compared to 4 in the intermediate and none with low D-dimer. There were no in-hospital

deaths, and the 3-month mortality rate in this cohort was 4%. *Summary/Conclusion.* We have shown that the level of D-dimer is related to the severity of PE assessed by various radiological, biochemical and clinical markers. Hence D-dimer could be of value as prognostic marker for the severity of PE. However, the low mortality rate precludes us from making conclusions regarding the predictive value of D-dimer on mortality. The prognostic value of D-dimer and its clinical significance need to be evaluated in properly designed prospective studies.

Table 1.

D-dimer mg/L	*Low 0.5-1.8 N=26*	*Intermed. 1.9-12.1 N=48*	*Hight 12.2-20 N=25*	*p-value*
PAOI>40%-N(OR)	1(1)	20(17)	18(64)	<0.0005
RV/LV ratio>1.0-N(OR)	2(1)	21(9)	16(21)	<0.0005
TNT*>0.01 ng/mL-N(OR)	1(1)	12(10)	12(34)	<0.0005

** TNT was measured in 67 patients; 18 in Low, 31 in intermediate and 18 in the high D-dimer caegory.*

0403

PREFERENCES AND WILLINGNESS-TO-PAY FOR TREATMENT OPTIONS IN PATIENTS ON ORAL ANTICOAGULANT TREATMENT (OAT): A DISCRETE CHOICE EXPERIMERNT

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Background. Vitamin K antagonists (VKA), have been used for more than 50 years and are still the only available oral anticoagulant drug. The main limits of VKA are: a narrow therapeutic window with relatively high incidence of minor bleeding; the need of strict laboratory monitoring, which means frequent dose adjustments; the interactions with foods/drugs, which requires attention to diet and other treatments. Due to their characteristics, VKA require both specific skill of physician and high compliance of patients. There is an increasing interest in the development of new anticoagulant drugs alternative to VKA: many new anticoagulant drugs are currently under clinical evaluation. *Aims.* Patients' preferences should be considered in the development of new therapeutic strategies. Aim of this study is to evaluate how patients who have to start anticoagulant treatment or are already treated with VKA in a large anticoagulation clinic perceive these attributes of new anticoagulant drugs as favourable, and what is their willingness to pay for getting these advantages. *Methods.* A discrete choice exercise, a technique for establishing the relative importance of different characteristics in the provision of goods/services, was applied to 97 consecutive stable patients on OAT and 140 consecutive patients during their first visit for starting OAT (127 male, 55%; mean age 63 SD=14). Patients had to choose between two different scenarios in 9 pair-wise comparisons. The attributes considered had previously been selected using an ad-hoc questionnaire administered to a sample of 20 patients and 6 physicians. The following attributes were selected: cost of treatment for the patient (€0 vs €15 vs €75/month), route and number of administrations, monitoring frequency, interactions with drugs/food (attention required vs not required), dose adjustment (required vs not required), minor bleeding (few vs no). Possible relationship between sociodemographic and/or clinical characteristics of the respondents and their preferences were evaluated. *Results.* The variable *cost* is determinant in patients choice. A monetary value can be assigned to each attribute. A significant monetary discrimination was reached for all attributes, except *interactions* and *dose adjustment*. Patients are willing to pay (WTP) per month: 79 for once/daily administration tablets vs one subcutaneous weekly injection; 41 for once/daily administration tablets vs two/daily administration tablets; 21 for once/monthly visit vs twice/monthly visits; 18 for each 6 month vs once/monthly visits; 23 for a dr ug without risk of minor bleeding. Stated the highest WTP for tablets once daily, patients on stable OAT showed a higher WTP for less *frequency of monitoring* and absence of *interactions*. Patients starting OAT showed a higher WTP for absence of *minor bleeding*. Younger patients (<55 ys) had a higher WTP for less *frequency of monitoring* and absence of *minor bleeding*. Older patients (>75 ys) had a higher WTP for absence of *minor bleeding*, and no concern about frequency of monitoring. *Summary/Conclusions.* The discrete choice experiment is a simply and well accepted method to asses patients' preferences, without medical or manufacturer interference. Except the cost, pharmaceutical formulation seemed to be the most relevant attribute that might make a choice of a new anticoagulant drug.

0404

THE VALUE OF THE DETERMINATION OF ACTIVATED PROTEIN C RESISTANCE (APC-R) IN HEMODIALYSIS PATIENTS

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Background. The genetic mutation of factor V Leiden which is characterized by an increased resistance to activated protein C (APC-R) is one of the most common inherited thrombophilia factors. Several studies suggest that about 4-8% of the general population is heterozygous for factor V Leiden. These rates are higher in some populations such as the northern Europeans. In Greece and Sweden some studies showed increased rates above 10%. Non molecular laboratory tests can demonstrate with high sensitivity (99.6%) and specificity (99.7%) the presence of this mutation. These tests can be performed in most coagulation analyzers and with low cost. On the other hand fistula or graft thrombosis is a common and costly complication in hemodialysis patients. Recent studies suggest that thrombophilia is associated with access thrombosis in these patients. *Aim.* The aim of this study was to establish the value of APC-R determination in hemodialysis patients by accessing the association between increased APC-R and vascular access thrombosis. *Methods.* In this retrospective study, 75 patients (36 men, mean age 63,2±10,9y and 39 women, mean age 62±12,2y) were selected from the hemodialysis Unit of the University Hospital, Iraklion Greece, between July 2003 and March 2005. The mean time on hemodialysis was 74,8±43,1 months. All patients were tested for antithrombin III, protein S, protein C, activated protein C resistance (APC-R), Lupus anticoagulant, antiphospholipid antibodies (panel), factors VIII and XI, homocysteine and lipoprotein(a). All participants were divided into two groups, those with access thrombosis (42 patients) and those with no access thrombosis (33 patients) and we assessed the prevalence of each thrombophilia factor to both groups. *Results.* Statistical analysis showed that among all tested thrombophilia factors only the presence of APC-R had a statistically significant association with thrombosis .Overall, nine patients (12%) had an increased resistance to activated protein C. All these patients had at least one episode of access thrombosis (100%). Univariate analysis to estimate crude (unadjusted) odds ratio showed a 2 times higher risk for access thrombosis in these patients: x2 =7.862, df=1, p=0,005, OR: 1.97 (95%CI: 1,56-2.49). In the previous statistic model no statistically significant differences were found after adjusting for sex, age, smoking habits, months in hemodialysis Hypertension, Diabetes Mellitus, Coronary Artery Disease, Cerebrovascular Disease, Peripheral Arterial Disease and Malignancy. *Conclusion.* This study revealed a statistically significant association between access thrombosis and increased APC-R in hemodialysis patients. This indicates that the determination of APC-R should be considered in these patients especially in populations with a high prevalence of factor V Leiden

0405

ALTERATIONS OF HEMOSTASIS AFTER LAPAROSCOPIC AND OPEN SURGERY

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Background. After tissue injury because of trauma or surgery, alterations of hemostasis are observed and there is a risk for postoperative thromboembolic complications. Laparoscopic surgery, by causing limited tissue injury, seems to be associated with a lower risk for thromboembolism than open surgery. However, this knowledge is inconclusive as it is based on a few studies, most often non-randomized. *Aims.* This prospective randomized study was conducted in order to detect potentially existing differences in activation of coagulation and fibrinolytic pathways between open and laparoscopic surgery. *Methods.* From January to September 2005 40 patients ASA1 and ASA2 were randomly assigned to undergo laparoscopic (group A n=20) or open cholecystectomy (group B n=20) by the same surgical and anesthesiology team. Demographic data were comparable. Blood samples were taken a) preoperatively, b) at the end of the procedure, c) 24 hrs postoperative-

ly and d) 72 hrs postoperatively. The following parameters were measured: platelets, soluble fibrin monomer complexes (F.S.test)), fibrin degradation products (FDP), D-Dimmers (D-D), fibrinogen (FIB), activated partial thromboplastine time (APTT), prothrombin time (PT). Thrombin-antithrombin III complexes (TAT) were measured at 24 hrs and 72 hrs postoperatively. Prothrombin fragment 1+2(F1+2) was measured at 24 hrs and 72 hrs postoperatively in 11 patients of group A and 13 patients of group B respectively. *Results.* Preoperatively, values of all haemostatic parameters were within normal limits in both groups. Immediately postoperatively, values of the coagulation markers TAT and F1+2 were significantly increased in the open surgery group as compared to the laparoscopic surgery group (p<0,05). Values of marker D-Dimmers were also significantly increased in the open surgery group (p<0, 01)immediately postoperatively and remained like that throughout the whole period of observation. Values of the coagulation marker FIB decreased slightly in both groups at 24 hrs postoperatively but there was a significant increase in the open surgery group as compared to the laparoscopy group (p<0,01) which remained like that thereafter. The APTT and PT values began to rise slightly in both groups but there was not observed a significant difference at any time between the two groups. The coagulation marker F.S. test became positive twice in the open surgery group starting immediately postoperatively and only once at 72hrs postoperatively in the laparoscopy group. Concentration of the fibrinolysis marker FDP was increased more in the open surgery group than in the laparoscopy group starting immediately postoperatively and this difference became significant 72 hrs postoperatively (p<0,05). No patient from either group suffered thromboembolism or abnormal bleeding as a postoperative complication. *Conclusions.* Open surgery as compared to laparoscopic procedures leads in activation of the clotting system of a higher degree than in laparoscopic surgery group implying thus a greater thromboembolic risk for patients undergoing open surgery. Subclinical fibrinolysis is also more profound at the open surgery group. Although of a lower degree, hypercoagulability is still observed in patients undergoing laparoscopic surgery and therefore routine thromboembolic prophylaxis should be considered.

0406

ROLE OF THE V617F MUTATION OF THE JAK2 IN PATIENTS WITH THROMBOSIS

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Background. Polycythemia Vera (PV) and Essential Thrombocytemia (ET) are Chronic Myeloproliferative Diseases (MPD) characterized by overactive hemopoiesis. Thrombosis is their main clinical complication. A single point mutation of JAK2 (Val617Phe) has been detected in most PV and in half the patients with ET. On the other hand, many patients suffer from thrombosis without an underlying cause. However, an underlying MPD has been demonstrated especially in patients with thrombosis in uncommon locations, such as in Budd-Chiari syndrome. The diagnosis of this underlying MPD is often very difficult and requires sophisticated methodologies. Before the advent of the JAK2 mutation, X-chromosome inactivation patterns and *in vitro* erythroid colony formation have been used. These methodologies are cumbersome and its use is restricted to some laboratories, but the investigation of the single point mutation (Val617Phe) of JAK2 is now readily available. *Aims.* The presence of the JAK2 mutation was assessed in a cohort of patients with thrombosis. *Methods.* A cohort of 309 patients with thrombosis were recruited from November 1997. Their DNA samples were analyzed by the allele-specific PCR methodology (1). DNA samples from 25 patients with PV were evaluated as positive controls. *Results.* In PV controls, 24 out of 25 cases showed the JAK2 mutation (96%). As for the patients with thrombosis, 1 out of the 309 patients with thrombosis was positive. This case was a 69-years-old male with 3 episodes of deep venous thrombosis and two of superficial venous thrombosis. His thrombophilia study was negative. This patient has been controlled in our department since 1997 and his Hb ranged from 160-168 g/l. Platelets and leukocytes were always normal. *Conclusions.* An obscure MPD is a very improbable cause of thrombosis. The investigation of the mutation V617P of the JAK2 gene should be reserved for special cases, such as patients with thrombosis in uncommon localization or patients with increased cell counts.

Reference

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0407

EVALUATION OF NEW COMMERCIAL ELISA KITS IN THE LABORATORY DIAGNOSIS OF ANTIPHOSPHOLIPID SYNDROME IN VIEW OF THE REVISED CLASSIFICATION CRITERIA OF THE ANTIPHOSPHOLIPID SYNDROME

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Since the publication of the 1999 Sapporo criteria for the classification of the antiphospholipid syndrome (APS) new clinical and laboratory insights have led to a recent update. These revised criteria now include testing for the presence of IgG and IgM β-2-glycoprotein I (β2GPI) with a positive titer being defined as higher than the 99th percentile of the normal population. aCL continues to be measured by a standardised ELISA. LAC positivity continues to be detected according to the ISTH guidelines. We have evaluated a newly developed Asserachrom® Antiphopholipid antibodies immunoassay line (Diagnostica Stago, Asnières, France) for the detection of antiphopholipid antibodies (APA) in a lupus anticoagulant (LAC) positive (n=137) and a LAC negative (n=134) population. The Asserachrom® APA Screen has been proposed to be used as a first screening assay for the qualitative detection of APA. Positive samples can be further investigated by the Asserachrom® APA IgG,M to determine the isotype and the quantitative antibody level. As 3 to 10% of APS patients are only positive for anti-β2GPI (Miyakis S., J Thromb Haemost, 2006) the Asserachrom® anti-β2GPI IgG and Asserachrom® anti-β2GPI IgM have also been proposed to be used in parallel to the Asserachrom® APA Screen. Despite that anti-prothrombin antibodies (aPT) are not included in the updated laboratory criteria they have been tested in this evaluation (Asserachrom® anti-prothrombin IgG,M). This new line of ELISA's uses monoclonal antibody based standardisation in accordance with the recommendations of the Standardisation Group of the European Forum on Antiphospholipid Antibodies for the APA, β2GPI assays. Imprecision characteristics performed with the included control material for all ELISA's were good, with coefficient of variation (CV) ranging from 4,9% to 13,9%. Cut-off values calculated with 99th percentile, as advised by the updated laboratory criteria, are higher than those currently proposed by the manufacturer (calculated with 97,5th percentile).The Asserachrom® APA Screen showed 2,6% false positive and 0,7% false negative results when compared with the Asserachrom® APA IgG,M which is acceptable. 49 patients out of 271 (18,1%) were positive for b2GPI antibodies. For 23 patients out of those 49, the Asserachrom® APA Screen was negative. This is in agreement with the above observation that the anti-β2GPI may be the only test positive (Miyakis S., J Thromb Haemost, 2006). 20 patients out of 271 (7,4%) had a positive titer for aPT antibodies, 40,0% of them (8/20) were negative with the Asserachrom® APA Screen. In conclusion, the Asserachrom® antiphospholipid antibodies line shows good performance characteristics and is a practical tool in the laboratory diagnosis of the APS. Own cut-off values should be calculated for each laboratory with the 99th percentile. As the anti-β2GPI may be the only test positive, the Asserachrom® APA Screen, the Asserachrom® anti-β2GPI IgG and the Asserachrom® anti-β2GPI IgM should be performed in parallel.

0408

DIFFERENTIAL EXPRESSION AND REGULATION OF PROTEASE-ACTIVATED RECEPTORS IN MONOCYTES FROM PATIENTS WITH ANTIPHOSPHOLIPID SYNDROME.

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Background. Patients with antiphospholipid antibodies (aPL) have an increased expression of Tissue Factor (TF) on monocytes and this can be one of the mechanisms leading to thrombosis. Depending on the cell type and the biological settings, TF seems to affect cellular properties through Factor VIIa-dependent proteolysis of factor Xa and thrombin, and subsequent activation of proteinase activated receptor PAR-1 and PAR-2. These PAR-activating proteases mediate responses that are critical for hemostasis and thrombosis, as well as inflammatory and proliferative reactions triggered by tissue damage. Yet, although PARs might play important roles in normal and pathological states, which protease(s) and PAR(s) functions in specific cellular processes remain unclear. In addition to its anti-inflammatory and immunomodulatory properties, statins have been shown antithrombotic effect, although the molecular mechanisms leading to this effect are not yet fully understood. *Objectives.*To investigate i) the aberrant expression of PARs in monocytes of patients with antiphospholipid syndrome (APS); and ii) the *in vivo* effects

of Fluvastatin on both PARs and TF expression in this experimental setting. *Methods.* Ten patients with APS and previous history of thrombosis received Fluvastatin (40 mg/day) for one month. Blood samples were obtained before treatment and after one and three month of treatment. Monocytes were isolated from peripheral blood mononuclear cells by magnetic depletion of non-monocytes. TF and PARs expression at both mRNA and protein levels were measured by real time RT-PCR, western blot, and flow cytometry. *Results.* Analysis of mRNA of the four PAR described to date in humans (PAR-1 to PAR-4) revealed that PAR1 was de most abundant member of the PAR family in the monocytes of APS patients. Significantly increased expression of PAR2 was also observed in relation to the control group. PAR3 expression was also demonstrated, but not significantly altered versus healthy controls. PAR4 expression was absent. Monocytes from all the APS patients studied showed significant inhibition of TF expression at both mRNA and protein levels after one month of Fluvastatin treatment (p=0.002). These levels then suffered a slowly recovery, although remained significantly lower than control values after three months of the end of the treatment. Interestingly, mRNA expression levels of PAR2 strictly paralleled this behavior in response to fluvastatin treatment. *Conclussion.* These results provide the first demonstration of increased PAR expression in monocytes from APS patients. Statins drugs indirectly downregulate thrombin generation at the cellular levels. Our study for explaining the anti-thrombotic properties of statins couples the downregulation of TF with inhibition of PAR expression. Thus, PAR blockade might also draws increasing attentions to its therapeutical applications for anti-thrombosis.

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0409

PROCOAGULANT FACTORS IN PATIENTS WITH CANCER

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Background. Clotting activation and thromboembolic manifestations are common features in patients with cancer. Tumor cells can directly activate the clotting through two procoagulants: tissue factor (TF) and cancer procoagulant (CP). *Aims.* the aim was to evaluate the levels of the TF and CP in patients with different tumors in order to: 1) to stablish an association between these markers and tumor localization, 2) to stablish a correlation between the levels of procoagulants and status of disease, 3) to evaluate if the treatment with chemotherapy induced some modifications on the levels of procoagulants, 4) to evaluate the possibility of using procoagulants as predictor factors in the development of thrombosis. *Methods.* Sixty-one patients with different types of cancer (lung, breast, digestive and genitourinary) and 20 normal controls were included. The activity of TF and CP was studied in blood serum. Statistical analysis of the data was performed by the two-tailed Fisher exact test. *Results.* The TF was increased in 72.5% and 0% (p<0.01) of cancer patients and normal controls, respectively. The PC was demonstrated increased in 88% of the cancer patients but in healthy controls it was increased in only 15% (p<0.01). The patients with genitourinary cancer presented the highest values of both procoagulants coinciding with a major prevalence of thrombotic events. The activity CP was found in 93% of patients with stages I and II but in patients with stages III and IV disease it was found in 85% (ns). They were not difference in levels of both procoagulants between the patients treated with chemotherapy and those with other treatments. *Conclusions.* TF and CP are elevated in patients with cancer. The highest values of both procoagulants are in the genitourinary cancer group in agreement with the greater presence of thrombosis observed in this group. A clinical follow up would be an important aspect to have a more clear idea on the potential value of these procoagulants and the tendency to develop thrombosis in patients with cancer.

0410

THROMBOPHILIC RISK FACTOR OF C46T POLYMORPHISM IN THE FACTOR XII GENE FOR VENOUS THROMBOSIS

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Background. Currently the dates in relation to thrombophilic risk factor of C46T polymorphism in the factor XII gene are contradictory; Tirado *et al.* are suggesting that the polymorphism itself is an independent risk factor for venous thromboembolism, another hand, Bertina *et al.*, in their study, are showing similar results for the frequencies of the C46Tgenotypes in patients and controls. *Aims.* The objective of this study is to define the prevalence of C46T polymorphism in the factor XII gene in health people and patients with venous thromboembolism and to establish his thrombophilic risk factor. *Methods.* A prospective study case/control we were included 516 subjects (219 patients and 297 controls). The patients with venous thromboembolism are diagnosed: 161 Deep Venous Thrombosis, 34 Pulmonary Embolism and 24 with both desease. The controls are healthy persons, blood donors, they were included in study voluntarily. The sex and age of the patients and the controls have a similar distribution. The detection of polymorphism factor XII 46C/T by PCR in real time, in liquid phase, in a LightCycler (Roche diagnostics) thermal cycler was made. The sequences of the allele primers are, forward: TTCTTCTgCTTCCAgTCCC and reverse: ATggCTCATggCAgTgATA. Stadistical methodology, the descriptive was made by groups in patients and controls; to estimate the risk by square-chi proof. *Results.* The results of prevalence of C46T polymorphism Factor XII gene in patients and controls are: patients CC 132 (60.3%), CT 75 (34.2%), TT 12 (5.5%) controls CC 200 (67.3%), CT 92 (31.0%), TT 5 (1.7%); in the next table are showed. The estimate risk to have got a venous thromboembolism event in relation to genotype TT of C46T polymorphism factor XII gene, with CI 95% is 3.6 (1.2;10.56) p=0.012. *Conclusion.* The allele homozygous T of C46T polymorphism in the factor XII gene is a thrombophilic risk factor for venous thromboembolic disease

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Table 1.

	Total	*CC*	*CT*	*TT*
Patients	*219*	*132 (60.3%)*	*75 (34.2%)*	*12 (5.5%)*
Controls	*297*	*200 (67.3%)*	*92 (31.0%)*	*5 (1.7%)*

0411

THE DIVERSE IMPACT OF SUBMAXIMAL EXERCISE IN THE FIBRINOLYTIC, COAGULATION AND INFLAMMATORY MECHANISMS IN PATIENTS NEVER TREATED FOR THEIR HYPERTENSION

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Background. Hypertensive patients are known for their atherogenetic background, based on haemostatic and inflammatory disorders, as well as for endothelial dysfunction. It is also known that exercise decreases cardiovascular risk in healthy individuals and in patients with coronary heart disease. *Aim.* The aim of the present study is to explore the effects of submaximal exercise in hypertensive patients focusing on the above mentioned disorders. *Patients and Methods.* Twenty (20) non-diabetic patients with newly-diagnosed essential hypertension (mean age 55+10 years, 11/9 male/female, mean office blood pressure 157/93 mmHg) participated in one 45 min submaximal exercise test on a bicycle ergometer. Blood samples were taken immediately before and after exercise and one hour after completion of the exercise. The following blood parameters were determined: a. Prothrombin time(PT),activated Partial Thromboplastin time (aPTT), D-dimers, Antihtrombin III(ATIII), Protein C(PrC),Prothrombin fragments 1+2 (PF1+2),Thrombin-antithrombin III complex (TAT) and factors VII and XII as indeces of activation of the coagulation cascade b.plasmin-a2 antiplasmin complex (PAP) as fibrinolysis index, c. platelet factor 4 (PF4) and β-thromboglobulin (β-TG) as indeces of platelet aggregation, d. soluble thrombomodulin (TM) and von Willebrand factor (vWf) as indeces of endothelial function e. White blood cell count (WBC),Fibrinogen (Fib) and free antigen of protein S (PrS) as inflammatory markers. *Results.* All patients completed the exercise test successfully. PAP significantly deviated throughout the whole exercise protocol (pre: 190.1+39 μg/Lt, exercise: 410.7+225 μg/Lt, after: 278.3+82 μ/Lt, $p<0.001$). A similar deviation was observed in WBC (pre: 6654±1118 /μL, exercise: 7283±1589/μL, after:9353±2310/μL, $p<0.001$) due to an increase in polymorphonuclear count. We noticed significant differences between pre- and immediately after exercise levels in aPTT (36±3.5sec vs 33±4 sec, $p<0.001$), Fib(361±68.4mg/dL vs 396.3± 65mg/dL, $p=0.001$), β-TG (107.8+52 IU/mL vs 143+52 IU/mL, $p=0.001$), TM (4.9+1.7ng/mL vs 4.2+1.4ng/mL, $p=0.009$) and vWf (114.6+48% vs 138.9+59%, $p=0.008$). Levels of aPTT(36±3.5sec vs 33.5±4 sec, $p=0.001$), factor VII (79.2+12% vs 74.7+10%, $p=0.001$) and vWf (114.5+48% vs 137.7+59%, $p=0.007$) differed significantly one hour after exercise as compared to pre-exercise levels. PrS was the only parameter that decreased significantly 1 hour after exercise as compared to immediately after exercise levels(96.8±17.7% vs 93±16%, $p=0.006$). No significant changes were observed in the levels of PT, D-d, ATIII, PrC, TAT, PF1+2, PF4, and factor XII. *Conclusions.* Submaximal exercise caused an exacerbation of the endothelial dysfunction and the inflammatory state, already existing in never treated hypertensive patients, as part of the atherosclerotic process. However, it seems that the prominent mechanisms were fibrinolytic and inflammatory rather than activated coagulation, since no hypercoagulable state was detected. Additional research, preferably directed towards inflammatory parameters, is required to determine the possible favourable effects of a chronic submaximal exercise programme in hypertensive patients.

Bleeding disorders

0412

COST AND QUALITY OF LIFE IN HEMOPHILIC PATIENTS WITHOUT INHIBITORS: THE COCHE STUDY

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Background. The adoption of modern treatment strategies in hemophilia care have significantly prolonged patients' life expectancy and efforts have being made to improve their health and wellbeing. *Aims.* our objective was to evaluate Cost of Care and Health-Related Quality-of-Life (HRQoL) of adult hemophilic patients without inhibitors. *Methods.* we conducted the naturalistic, multicenter, longitudinal Cost Of Care of HEmophilia (COCHE) study involving >18-year-old patients sequentially enrolled at 23 Italian Hemophilia Care Centers. Information collected was on socio-demographic and clinical data, resource absorption, HRQoL, treatment satisfaction. *Results.* 232 patients were enrolled (median age=34.3, 18-74), 86.6% with hemophilia A, 72.4% severely affected. At enrolment 81.0% of patients had chronic hepatitis C, 25.0% hepatitis B, 15.9% HIV infection. Most of the patients (87.8%) had some or severe orthopedic problems. The total World Federation of Hemophilia Orthopedic Joint Score (OJS, the higher the score, the worse the functioning) was 0-5 in 74 patients (32.2%), 6-16 in 81 patients (35.2%), 17-66 in 75 patients (32.6%). During the follow-up 81% of patients bled at least once, with a median of 1.44 episodes per month. One to 6 target joints were present in 56.5% of patients. Compared with general population HRQoL appeared compromised in physical sphere of health but not significantly reduced in the mental one: the mean (SD) SF-36 Physical Component Summary (PCS, the higher the score, the better the HRQoL) score was 42.5 (10.2) and Mental Component Summary (MCS) score was 45.8 (8.5). The mean (SD) EQ-5D Visual-Analogue-Scale (EQ-VAS), evaluating general HRQoL, was 66.2 (18.4). The major direct cost driver from the Italian National Health Service's perspective was attributable to treatment with coagulation factor concentrates (96.6% of total direct cost), corresponding to 8,500 /patient/month. These data wer e significantly different according to some patients' characteristics, such us severity of orthopaedic impairment. To date, the median hemorrhages per month in joints and muscles was 0.6, 1.3, 1.5 in first (OJS=0-5), second (OJS=6-16) and third (OJS=17-66) severity group, respectively. The mean PCS was 51.8, 42.9, 35.4, the mean MCS was 48.9, 46.0, 43.7, the mean EQ-5D VAS was 80.0, 69.5, 60.0 in the first, second and third severity group, respectively. Frequency of patients receiving a regular continuous replacement treatment for >16 weeks was: 24.3% in first, 34.6% in second and 40.4% in the third severity group. Consequently, cost of treatment increased with OJS increase: patients scoring 0-5 cost on average 6,800€/patient/month, those scoring 6-16 cost 8,000 €/patient/month, those scoring 17-66 cost 11,000 €/patient/month. Discussion: Cost of hemophilia care have been increasing because of a wider use of more effective but more costly treatment strategies. More severe clinical status leads to worse wellbeing and to the need to implement regular continuous replacement treatments. The use of strategies aimed at preventing long term consequences such as arthropathy can have important repercussions on patients' health status, on their wellbeing and on a more efficient resources consumption.

0413

PROPOSED GUIDELINES FOR ANTICOAGULATION DURING CARDIAC SURGERY USING ARGATROBAN

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Background. Anticoagulation during cardiac surgery is essential and almost always done with heparin. Some patients with contra-indications to heparin, like allergy or heparin-induced thrombocytopenia, require surgery. Knowledge about the use of alternative anticoagulants to heparin, like direct-thrombin inhibitors (DTI) is limited. Some major issues with DTI during surgery such as, bleeding complications, optimal laboratory monitoring, and safety have not been systematically addressed. In these situations, physicians have to make clinical decisions based on personal or anecdotal experience. Previously, we have described our experience with the direct thrombin inhibitor argatroban for anticoagulation during cardiac surgery. Here, we systematically

review the information on all published cases of argatroban use during cardiac surgery in adults. *Aim.* The aim of this study is to develop guidelines for the appropriate use of argatroban during adult cardiac surgery. *Methods.* The information on all reported adult cases of argatroban use during cardiac surgery was reviewed. This analysis focused on patient characteristics, type of surgery, argatroban dosing schedule, monitoring of anticoagulation, morbidity and mortality. *Results.* Twenty-one cases have been reported. Fifteen patients underwent off-pump surgical procedures with the argatroban initial dose 5 mcg/kg/min infusion adjusted to maintain an activated clotting time (ACT) range between 200-300 s. Three intra-operative thrombi occurred in two patients when the ACT was less than 280 s. None had coagulopathy. Six cases reported the use of argatroban during CPB dosed with a bolus of 0.1-0.3 mg/kg followed by an infusion of 5-10 mcg/kg/min to keep the ACT greater than 400 s. Intra-operative thrombotic complications were not reported in this group; however, one clot in the pump was noted after the procedure when the ACT was between 300-350 s. All six cases required larger volumes of peri-operative blood products and 3 had severe coagulopathy. Of the 21 cases, 7 had an indication for continued anticoagulation following surgery. Four cases did not report further use of argatroban after surgery. Three patients received argatroban after surgery without complications. *Conclusions.* Argatroban, with ACT monitoring, can be safely used for anticoagulation during cardiac surgery using the following proposed guidelines. We recommend an ACT level of greater than 300 s for off-pump cardiac surgery and greater than 400 s for CPB. It would be advisable to use an arbitrary upper limit of ACT for both on and off-pump procedures to prevent severe coagulopathy. ACT monitoring seems to be a clinically reliable test to predict coagulation status in patients undergoing cardiac surgery with argatroban and should be checked often (i.e. every 15 minutes) to allow for proper adjustments of the dose. Prospective studies to evaluate the optimal dose and monitoring effect of this agent during cardiac surgery should be supported.

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0414

PILOT STUDY TO ESTABLISH PREFERENCES TOWARDS COAGULATION FACTOR CONCENTRATES USED TO TREAT HAEMOPHILIC PATIENTS WITH INHIBITORS

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Background. Haemophilia is a very expensive disease. This situation becomes extreme when patients develop inhibitors that compromise the effectiveness of treatment, with potential increase of morbidity and mortality. Treatment of haemophilia is the result of interactions between patients, physicians, pharmacists and budget holders, each carrying their own set of preferences. *Aims.* A pilot study was conducted to identify which characteristic of coagulation products are considered more important to treat patients with inhibitors: these characteristics will be included with a price proxy characteristic in a Discrete Choice Experiment, with the objective to elicit preferences and willingness to pay towards treatments of patients with inhibitors. *Methods.* 8 characteristics were identified during focus groups with patients and clinicians and rated from 0 (not important) to 10 (very important) by 35 people (adult patients, caregivers, physicians, pharmacists). *Results.* the following median (mean) scores were found: *viral safety*: 10 (8.9); *time to stop bleeding*: 9.5 (9.0); *risk of anamnestic response*: 9.0 (8.5); *possibility of undergoing major surgery*: 9.0 (8.8); *regular use in prophylaxis*: 9.0 (8.4); *time to pain recovery*: 9.0 (8.3); *number of injections to stop bleeding*: 8.0 (7.9); *time to prepare and give/have the injection*: 7.0 (6.6). All groups of respondents considered as more important *viral safety*, *possibility of undergoing major surgery*, *risk of anamnestic response*, *time to stop bleeding*, while *time to prepare and give/have the injection* was considered the least important. Different preferences were attributed to *time to pain recovery*, considered more important by patients; *regular use in prophylaxis*, considered more important by caregivers. *Conclusions.* viral safety and effectiveness are considered as the most important characteristics in the treatment of haemophilic patients with inhibitors. Different levels of preferences are present between patients, or their caregivers, and physicians. Understanding these differences is important to guide optimal therapeutic strategies in patients with inhibitors.

Table 1. Proposed guidelines for argatroban use during cardiac surgery in adults.

	Off pump cardiac surgery	*On pump cardiac surgery*
Argatroban dose	5 mcg/kg/min infusion Adjust according to ACT	0.1 mg/kg bolus followed by 5 mcg/kg/min infusion Adjust according to ACT
Target ACT	>300s	>400s
Monitoring	ACT every 15 min	ACT every 15 min
Low ACT level	300 s	400 s
High ACT level	500 s (arbitrary level)	600 s (arbitrary level)
Risks	Intra-operative thrombi formation with ACT < 300s	Coagulopathy with high ACT levels

0415

PRELIMINARY RESULTS FROM THE EUROPEAN ESCHQOL FIELD STUDY

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Background. The ESCHQoL Study is a retrospective/prospective prevalence based cohort study of clinical, quality of life and health economic outcomes in haemophilia treatment in Europe and is sponsored by the EU. 1732 patients with haemophilia from 4 years on have been recruited from 19 European countries, out of them 78% have been enrolled in the study. The ESCHQoL Study consisted of a pilot testing phase where the study procedure was feasibility tested in 70 patients and a field testing phase in which more then 1.300 haemophilia patients participated. *Aims.* Objectives of the study were a) to make validated instruments available for the assessment of patients' health status, quality of life and health care and its cost on an European basis, b) to identify models of health care of haemophiliacs in terms of clinical characteristics, their possible costs and impact on quality of life, c) to provide policy recommendations for optimal care of haemophilia patients based on clinical, quality of life and health economic information. *Methods.* In the ESCHQoL Study patients with haemophilia were asked to complete a comprehensive questionnaire concerning socio-demographic (such as age, gender), psycho-social (such as quality of life, coping) and health economic (such as days lost of work, costs of care) information and to fill in a diary during a 6-months period. In parallel, clinical information concerning *bleeding history*, *inhibitor history*, *concomitant disease*, *arthropathy assessment*, *surgery*, *treatment modality*, *type of product* and *medical visits* was obtained from physicians. *Results.* In the pilot testing 70 patients (41 adults, 29 children) were included from the steering committee countries (Italy, Germany, Romania, Hungary, Sweden, U.K.) and France. According to the feedback evaluation, patients and parents found the questionnaire good and interesting, but many said it was too long and repetitive. All respondents found the questions relevant for haemophilia. The extended completion time (55 min) led to the decision to shorten the questionnaire for parents and adults. The pilot testing questionnaire was divided into two parts in order not to lose information, where one part was administered at baseline the other part was given at follow-up. Suggestions from physicians were implemented in the medical documentation. In the field testing 1.343 patients (931 kids, 1.412 adults) were enrolled from 19 countries. 87% had haemophilia A and were severely affected (72%). In 11% inhibitors occurred and one third of the patients received prophylactic treatment. 45% of the patients suffered from chronic pain and 40% reported target joints. Viral infections were found in 42% of the patients (hepatitis C) and 8% for HIV. 5% of the patients underwent an orthopaedic surgery. *Conclusions.* The feasibility testing of

the study documents revealed that the original questionnaires had to be modified for the field testing. Preliminary results of the field testing revealed differences between countries concerning clinical status and treatment modalities and their impact on costs and quality of life. These results underline the importance of the aim of the ESCHQoL *Study*. to compare QoL outcome of haemophilia care in Europe in order to recommend future improvements.

0416

INTRACRANIAL HEMORRHAGE IN HEMOPHILIACS RECEIVING NO PROPHYLACTIC REPLACEMENT THERAPY

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Background. Intracranial hemorrhage (ICH) in hemophiliacs is the primary cause of death through bleeding. The mortality risk associated with ICH was 70% before the introduction of cryoprecipitate; it decreased to 25-30% thereafter but remained still very high. *Aims.* Analysis of the frequency, type, severity, and consequences of the ICH in hemophiliacs from Romania, receiving no prophylactic replacement therapy. *Methods.* The study was conducted on a cohort of 212 hemophiliacs, 180 with hemophilia A, and 32 with hemophilia B, 51.89% with severe disease, 50.48% with sporadic disease. The period of the study was 16 years (1990-2005). *Results.* Intracranial hemorrhage was registered in 18/212 (8.49%) of the studied patients, being a very rare bleeding (0.46% of the registered bleedings). ICH was the manifestation at diagnosis in 6.92% of the patients. It appeared mostly in case of severe hemophilia (77.78%), at different ages. Head trauma registered in 46.49% of cases in patients aged between1-6 years, was complicated by ICH in 9.18% of the cases. Except for the neonatal period, the risk for ICH after head trauma was higher in school aged boys (10.71%). ICH appeared even after minor head trauma (14.56%), sometimes being apparently spontaneous. Birth trauma represented the most important risk factor for ICH, determining 7/18 (38.39%) of the ICH cases, respectively 3.3% of the patients, all with severe hemophilia. ICH was rapidly recognized and treated in only one case, although in five cases hemophilia has been diagnosed in the family before. Four patients with ICH were treated in our clinic, one being also operated, and neither has complications. The long term complications of the ICH (neurological, sensorial and psychological sequelae) were very frequent (72.22% of the ICH cases), generally extremely severe (epilepsy-76.92%, hydrocephalus-15.38%, palsy-15.38%, blindness-23.08%, mental retardation-30.78%, aso.), and usually associated. The risk for permanent damages was even higher in case of ICH associated to birth trauma: 91.67%. No deaths through ICH were registered in the period of study. *Conclusions.* ICH is a very dangerous bleeding in hemophiliacs receiving no prophylactic replacement therapy, being the cause of the most serious long term complications. The prevention, early recognition, and correct replacement treatment in case of ICH are essential in order to reduce their consequences. Many hemophilia centers developed special protocols for the prevention of ICH associated to birth trauma or head trauma. Although the prophylactic replacement therapy proved not to be sufficient in order to prevent ICH, the efficiency of early home therapy in case of head trauma is well documented.

0417

PREVALENCE OF ANTI-HLA AND ANTI-GPIIB/IIIA ALLO-IMMUNIZATION IN PATIENTS WITH GLANZMANN THROMBOASTHENIA: EXPERIENCE OF A SINGLE CENTER

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Background. Platelet transfusions, the main therapy of Glanzmann Thromboasthenia (GT), can induce an allo-immunization against HLA antigens and GPIIb/IIIa complexes, with a possible reduction of efficacy of subsequent treatments. *Aims.* To investigate the development of allo-antibodies anti-HLA antigens and anti-GPIIb/IIIa complexes in GT transfused patients, and evaluation of efficacy of replacement therapy. *Patients and Methods.* From 1975 onwards, we have followed 17 GT patients; 12 type I, 3 type III, 2 not classified; 8 men, 9 women; median age at diagnosis 9.8 years (range 1-44.5); median age at the time of this study, 35.5 years (range 23.6-68.5). Our patients showed at least once in their life the following symptoms: 10/17 epistaxis; 5/17 gastrointestinal hemorrhage; 5/17 oropharingeal hemorrhage; 4/17 muscle hematoma; 2/17 bleeding for traumatic injury; 2/17 hemarthrosis; 2/17 hematuria; 1/17 intracranial hemorrhage; 1/17 hematothorax; 1/17 otorrhagia. Five/9 women experienced meno-metrorrhagia. Ten major and 22 minor surgical procedures have been performed. Two spontaneous deliveries and 3 cesarian sections with 5 live births have been observed; moreover, 2 abortions occurred, 1 spontaneous and 1 voluntary. Globally, 9/17 patients have been transfused with platelets and red blood cells (RBC); 5/17 only with platelets; 2/17 only with RBC. One patient has never been transfused. Platelet transfusions have always been hemostatically effective. Fifteen/16 transfused patients have been investigated for allo-antibodies, anti-HLA and anti-GPIIb/IIIa. *Results.* The positivity for allo-antibodies has been demonstrated in 4/15 patients (27%): isolated for anti-HLA in 2; isolated for anti-GPIIb/IIIa in 1; combined in 1. *Conclusions.* The prevalence of allo-immunization (27%) is inferior to recent literature data (50%). While positivity for anti-HLA (3/15, 20%) agrees with the recent literature data (22%), positivity for anti-GPIIb/IIIa (13%) is inferior (35%). Presence of allo-immunization did not compromise the efficacy of platelet transfusions.

0418

GENETIC ANALYSIS OF THE COAGULATION FACTOR VIII AND IX GENES IN HUNGARIAN PATIENTS WITH HAEMOPHILIA

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Haemophilia A (HA) and haemophilia B (HB) are common X-linked bleeding disorders resulting from the inherited deficiency of coagulation factors VIII (fVIII) and IX (fIX), respectively. Female relatives of patients with haemophilia may be carriers and many of them request carrier status determination. Previously, large gene inversion detection in HA patients and linkage analysis using intragenic polymorphisms in HB and in inversion-negative HA patients were used for carrier and prenatal diagnosis in our laboratory, but in some cases linkage analysis had limitations. A high number of different mutations can be identified by direct sequencing of the fVIII and fIX genes, which is now the preferred method for genetic analysis. The aim of our study was to provide precise carrier status determination in affected families. Upon selection of 16 severe HA-patients without intron 1 and 22 inversions and 20 HB-patients we decided to identify the disease causing mutations by sequencing the promoter and the exons with flanking regions of the fVIII and fIX genes using dideoxy chain-termination method. Previously unpublished mutations were identified in 10/16 (62.5%) of the HA patients examined. Distribution of the novel mutations in the fVIII gene are the following: 3 novel missense [370A>C (Lys48Gln), 1883T>C (Leu552Pro), 2084G>A (Gly619Asp)], 2 nonsense [430G>A (Trp68 STOP), 3839T>A (Leu1204STOP)] mutations, 3 small deletions [2769delG, 1473delC, 4218delG], one splice site variant [A>T -2 IVS22], and one ins/del mutation [nucleotide 6736-6754]. Beside these new mutations, six published point mutations (4 missense, 1 nonsense and 1 small insertion) of the fVIII gene were also found . Among 20 HB-patients, one novel [g.10443A>T (Asp64Val)] and 17 published fIX gene mutations were found. The new mutation was detected in two unrelated Hungarian families sharing identical haplotypes. Our results further confirm, that HA and HB can be caused by a wide variety of point mutations and indicate that regarding HA, significant proportion of novel mutations can be identified upon sequencing previously untested populations.

0419

RELATIONSHIP OF PAI-1 4G/5G POLYMORPHISM AND BLEEDING RISK ASSOCIATED TO CARDIAC SURGERY

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Introduction. Plasminogen activator inhibitor-1 (PAI-1) is an important inhibitor of the fibrinolytic system. Several studies have pointed out that patients with the 4G allele of PAI-1 polymorphism (homozygous 4G/4G and heterozygous 4G/5G) have a higher level of PAI-1 in plasma, when compared with homozygous 5G/5G. *Aims.* The primary target of this work was to analyse the influence of PAI-1 genotype on the bleeding risk of patients undergoing cardiac surgery with cardiopulmonary bypass. *Methods.* We have studied 26 cardiac surgery patients in our center (15

men and 11 women; median age 67 years, range 40-85), distributed according to PAI-1 genotype (5G/5G versus 4G/5G or 4G/4G). Not to interfere with the PAI-1 plasma concentration, patients receiving tranexamic acid during the intervention were excluded. Among others, main recorded data included total hemorrhage volume, corporal temperature, total dose of heparin and protamin, prothrombin time (PT) and activated partial thromboplastin time (APTT), fibrinogen, d-dimer, antithrombin, PAI-1, tPA, lactic acid, hemogram and transfusional requirements. Four moments were chosen to analyse the different parameters: basal, arrival to intensive care unit, after 4 hours of arrival and after 24 hours. Laboratorial and statistic methodologies are explained. *Results.* Patients carrying the PAI-1 4G allele (both homozygous and heterozygous) presented a smaller bleeding risk with respect to the homozygous 5G/5G, with significant differences in bleeding at the first 4 hours (332±223 vs. 846±519 mL; p=0.002) and 24 hours after arriving at the intensive care unit (771±446 vs. 1379±582 mL; p=0.016). This smaller hemorrhagic risk correlates with significantly elevated PAI-1 levels at the moment of arrival at the unit in the patients carrying 4G allele (120,3±103 vs. 36,9±7,7 ng/mL; p=0.019), and also higher levels of antithrombin (p=0.016), PT (p=0.019) and fibrinogen (p=0.027), and a higher corporal temperature (p=0.011). We did not find significant differences between both groups of patients for the rest of analysed parameters; all the results are exposed. *Conclusions.* Some authors have studied the relationship between PAI-1 4G/5G polymorphism and the risk of ischemic events (mainly coronary disease and ictus), but few reports exist that clinically correlate this polymorphism with hemorrhage. Our work demonstrates that patients undergoing cardiac surgery who are carriers of the PAI-1 4G allele, may have a significantly lower bleeding risk in the first 24 hours after surgery, when they are compared with homozygous 5G/5G. Although preliminary, of these findings it is possible to deduce that patients carrying the 4G allele could not need antifibrinolytics, and thus, to contribute to avoid possible thrombotic complications.

0420

FACTOR XI DEFICIENCY AND POST-PARTUM HAEMORRHAGE: BLEEDERS AND NON-BLEEDERS!

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Management of pregnant women with Factor XI deficiency poses a challenge to the clinician because of the variable bleeding tendency and risks associated with factor replacement. Factor XI deficiency is an uncommon bleeding disorder with an autosomal inheritance. The gene frequency varies, comprising only 7% of bleeding disorders on our local data base in the East Midlands (UK). In heterozygotes, there is mild or moderate reduction in Factor XI level, between 20-70 u/dL. Homozygotes or compound heterozygotes have severe reduction in levels, often < 1u/dL. There is no clear correlation between FXI level and bleeding tendency and it is generally trauma or surgery-related. There are few data on pregnancy complications and FXI deficiency. Our study objectives were to assess pregnancy outcome with respect to miscarriage rate and post-abortal bleeding, and post-partum haemorrhage. Since symptoms of bleeding disorders can be difficult to assess objectively, we applied the criteria employed by Bolton-Maggs. Two haematologists independently assessed each set of case-notes and classified each patient into either *bleeder* or *non-bleeder* groups. 34 women were identified on the local data-base. Thirty'one had moderate or mild deficiency, while 3 had severe reduction in FXI levels. The patients were evenly divided between *bleeders* (B) and *non-bleeders* (nonB), with 18 and 16 respectively. In 2 cases, the women had not undergone surgical or dental challenges, and were classified as *non-bleeders* in the absence of menorrhagia +/or mucous membrane bleeding. They had a total of 109 pregnancies, with 79 live births. Pregnancy and delivery was uneventful in the majority of cases, 71% overall (76% nonB; 65% B). Of those pregnancies resulting in a live birth 80% were uneventful (92% nonB; 72% B). The local incidence of PPH is 5%. The total number of instances of PPH in our study was ten (13%), 9 primary and 1 secondary. This increased incidence of PPH was statistically significant, with a p value of 0.029. All but two episodes occurred in the group of women with increased bleeding tendency. Of the women in this group, PPH occurred twice in 2 patients and once in 4 women. When the incidence in *bleeders* was compared to that of *non-bleeders* there was a highly significant difference, (p 0.000001).In this study, 10 women suffered a total of 13 spontaneous miscarriages. One further woman had a total of 15 miscarriages and was excluded from this analysis. The total rate of miscarriage between 8 and 13 weeks, locally, is 10%. Eleven of the miscarriages in our study were within this time period, giving a miscarriage rate of 10.6%. The twelfth miscarriage occurred at 22 weeks, in a young woman who had ruptured her membranes at 20 weeks. The cases appeared evenly divided between the two groups However, significant post-abortal bleeding was noted in 2 cases, both *bleeders*. In summary, although uneventful for the majority of women, factor XI deficiency caused pregnancy complications for a subgroup with a bleeding diathesis. Further studies are required to define the underlying factors of this group.

0421

USE OF LOW DOSE RECOMBINANT ACTIVATED FACTOR VII INFUSION FOR TREATMENT AND PROPHYLAXIS OF BLEEDING EPISODES IN SEVERE FACTOR VII DEFICIENCY

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Introduction. Severe factor VII deficiency is a rare bleeding disorder which can be treated with fresh frozen plasma, prothrombin complex concentrates and plasma derived factor VII. Recombinant activated factor VII (rVIIa, Novoseven) is now licensed for the treatment of factor VII deficiency at a recommended dose of 15-30 micrograms per kilogram (mcg/kg) by bolus injection every 4-6 hours. Correlation is poor between factor VII activity and haemostasis, but levels of 10-15% of normal are generally sufficient to achieve haemostasis. Activated factor VII makes up 1% of the total and in combination with tissue factor is the initiator of coagulation. The licensed dose of rVIIa raises plasma levels of factor VIIa far above the physiological norm. The half-life in plasma is short (2.30-2.97 hours) requiring frequent bolus injections. Infusions of low dose rVIIa seem attractive as it abolishes peak and trough levels and avoids exposure to blood borne viruses and prions. *Patients and methods and outcomes.* Six patients (3 children and 3 adults) with severe factor VII deficiency (levels <1-4%) were treated with infusions of rVIIa totalling 13 episodes (7 bleeding episodes and 6 elective invasive procedures) between July 2003 and January 2006. Five patients are included in Table 1. The sixth patient presented at 5 weeks old with an intracranial haemorrhage.

Table 1.

Patient	*Age/Sex*	*Dose Mcg/kg/24 h*	*Length Days*	*Procedure*
1	2.5 m M	300	4	Peri-anal tear req transusion
2	8 y M	75	1	Multiple dental extractions
3	22y F	17.1	3	Termination of pregnancy
4	22 y F	19	3	Normal vaginal delivery
	23 y	19	4	Normal vaginal delivery
5	29 y F	17.1	1	Multiple dental extractions

She was treated with a bolus of 60 mcg/kg followed by an infusion of 250 mcg/kg/24 hours for 24 days. During this time she underwent insertion of an extra-ventricular shunt and of a central venous catheter. There was no excessive bleeding. She has required six further infusions (2- 7 days duration) for hip bleeds, gastro-intestinal bleeding and central venous catheter changes with no evidence of excessive bleeding, at doses of 150-250mcg/kg per 24 hours. In emergencies the patients were given a bolus of rVIIa (60 mcg/kg) immediately followed by an infusion of rVIIa (1.2 mg diluted in 24 mL 0.9% saline) until the bleeding was controlled. The dose 1.2 mg in the infusions is dictated by this being the smallest vial currently available. In elective procedures an infusion of rVIIa (17.1-300 mcg/kg/24h) was commenced 2-4 hours prior to procedure. *Results and Discussio*n. Our results show there were no episodes of increased blood loss over the expected for the procedure. There were no episodes where extra doses of rVIIa or other treatment were required. Therefore we conclude it is feasible, safe and effective to use low dose rVIIa infusions in both emergency and elective situations. Using doses of 20mcg/kg per 24 hours via an infusion pump appears to be as effective as the licensed dose of 60-180 mcg/kg per 24 hours in adults and may reduce the theoretical risk of thrombo-embolic phenomena. The reduction in dose represents a large cost saving and requires less medical and nursing time refilling the infusion every 24 hours. It may be possible to further reduce the dose given in paediatric patients with smaller vial sizes or if the rVIIa is shown to be stable for a longer period once reconstituted.

0422

EVALUATION OF BONE MINERAL DENSITY IN CHILDREN WITH HEMOPHILIA: MANSOURA UNIVERSITY CHILDREN HOSPITAL (MUCH)EXPERIENCE: MANSOURA, EGYPT

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*Background.*Patients with hemophilia may be at risk for developing reduced bone mineral density for a number of reasons such as recurrent hemoarthrosis and immobilization. *Aim of the Work.* To assess the bone mineral density(BMD)in children with hemophilia and, to correlate bone mineral density with findings regarding the joint disease (hemophilic arthropathy). *Patients and Methods.* Thirty hemophilic patients aged 4.97+3.64 years and 30 control healthy individuals (had no joint disease) aged 5.09+3.64 years were selected from the hematology unit and outpatient clinic of MUCH respectively. Anthropometric measurements was done to all cases. Z score was used for weight, height, and Body Mass Index(BMI). Joint evaluation for hemophilic patients and controld was done using Colorado PE-0.5: Half Point Instrument before using Dual Energy X-ray Absorptiometry(DEXA). DEXA scanning was performed to all hemophilic patients and controls focusing on L2-L4. *Results.* There was no significant difference between hemophilic patients and controls as regard anthropometric measurements and their z-score. There was a significant difference between hemophilic patients and controls as regard BMD and BMD z-score (p= 0.05, 0.003) respectively. There was a significant difference between severe hemophilic patients (factor level assay less than 1%) ans controls as regard BMD and BMD z-score p=0.01, 0.001) respectively. Also, in hemophilic patients, there was an inverse significant correlation between joint evaluation scores and BMD z-score (r=-0.365, p=0.04). *Conclusions.* Children with hemophilia could have reduced bone mineral density compared with age and gender matched controls. This reduction in bone mineral density was independent on difference in age and body size.Children with more established hemophilic arthropathy exhibited the lowest BMD and BMD z-score. *Recommendations.* 1. Early detection of osteopenic hemophilic children using DEXA scanning. 2. Bisphosphonates plus calcium for hemophilic children with reduced bone mineral density. 3.Evaluation of the effect of on demand versus prophylaxis replacement therapy in hemophilic patients on BMD and hemophilic arthropathy.

0423

CLINICAL AND LABORATORY PECULIARITIES IN CHILDREN WITH VON WILLEBRAND DISEASE

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Von Willebrand disease (vWD) is the most widespread (frequency 1%) hereditary form of hemorrhagic diathesis after hemophilia. The main causes of bleeding in patients with vWD are quantitative and qualitative alterations of von Willebrand factor (vWF) in plasma, although vWF of platelets has an influence on severity of disease symptoms. The prominence of the bleeding varies due to complicated pathogenesis and variability of the forms. The aim of this study was to establish the clinical and laboratory peculiarities of vWD in children. *Materials and Methods.* We have assessed hemostasiogramms in 150 children with vWD aged from 6 mo to 14 years, including 35 children with parents (13 fathers and 22 mothers). *Results and discussion.* vWD was more frequent in girls (60%). In majority of patients diagnosis was established during initial examination, in 45% during the second examination. Among patients were 2 infants, 8 children of 1-3 y.o., 11 of 4-6 y.o. and 14 patients aged from 7 to 14 years. An evaluation of patients genealogy has allowed to confirm familial character of the disease in the majority of patients 84%, an autosomal-dominant type of inheritance was observed in 60%. The first symptoms of the disease were appear in earlier childhood (44%) and in childhood (52%). The main symptoms of the hemorrhagic syndrome were: nasal bleeding (92%), skin hemorrhages (40%), post-operative and post-traumatic bleeding (36%), subcutaneous hemorrhages (18%), tooth (18%) and gingival (6%) bleedings. Metrorrhagias were leadings symptoms in 11 girls aged 12-14 years. Intensity of the bleedings was moderate in the majority (68%) of children. Disease course was characterized with periods of bleeding and remission of various duration. The frequency of bleeding episodes was: monthly in 12%, several times per year in 24%, annually in 36%, more rare in 28%. Laboratory investigations were started from routine test: bleeding time (BT) and number of platelets (P). BT was increased in 68% of patients, P was 150-200 thousands/L in all patients. Diagnosis of vWD was established on results of cogulological investigations: vWF, coagulation factors levels and platelet functions. Platelet adhesion to glass (normal value 30-40%) was decreased: in 12% of patients was 0%, in 20% was 1-10%, in 25% - 10-20% and in 40% of patients was 20-30%. Ristomycin-induced (1,2 mg/mL) platelet aggregation was decreased in al patients: in 72% was up to 17 sec, in 28% of patients up to 20 sec (normal value 3-10 sec). Activated partial thromboplastin time (APTT) was prolonged: in 48% of patients to 55-60 sec, in 28% to 61-60 sec, in 16% to 71-80 sec and more than 80 sec in 8% of patients. 50% decreasing of factor VIII was noted in 50% of patients: in 27% to 20-50%, in 23% of patients to 11-20%. The most accurate indicator in diagnosis of vWD is estimation of blood vWF level. In the majority of children decreasing of this indicator was observed, and levels of vWF were: 0-20% in 12% of patients, 20-40% in 12%, 40-60% in 36% and 40-60% in 40%. So, in the majority of cases vWD type I (plasma vWF protein decreasing) and type III (complete absence of vWF protein) were noted. Almost normal vWF level (60-80%) does not exclude vWD type II. In this case diagnosis can be established by vWF multimeric structure investigations, which not available in our laboratory. Same clinical and laboratory changes were observed in both children and parents in 28 cases, in 7 cases more prominent changes were noted in parents in comparison with children. Thus, von Willebrand disease is one of the most widespread hemorrhagic diatheses with autosomal-dominant or, more rare, autosomal-recessive inheritance. Variability of the clinical and laboratory signs is typical for vWD. vWD can be suspected in cases of familial bleedings in both genders. Relapsing spontaneous nasal bleedings-more frequent symptom of the disease. In patients with vWD vascular-platelet hemostasis alterations are seen: bleeding time increasing, ristomycin-induced platelet aggregation decreasing. Alterations of coagulative chain of hemostasis are characterized by factor VIII activity decreasing and moderate increasing of activated partial thromboplastin time. Most accurate investigation in diagnosis of von Willebrand disease in von Willebrand factor level estimation.

0424

PLATELET FUNCTION TESTING IN URAEMIC PATIENTS

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Background. Chronic renal failure (CRF) is associated with excessive bleeding. Platelet dysfunction is probably the most consistent and important feature, particularly platelet-platelet and platelet-vessel wall interactions. The skin bleeding time (SBT) is the best-established predictor of bleeding in uraemic patients. However the SBT suffers from poor reproducibility and accuracy. Additionally the common SBT kit (Simplate II) has been withdrawn from sale in Australia. Several newer rapid assays of platelet function are able to provide a means of assessing primary haemostasis, but have not been specifically assessed in uraemic patients. *Aims of the study.* A pilot study to examine various *in vitro* assays to assess haemostatic defects in uraemic patients. These assays include platelet function analyzer (PFA-100), cone platelet analyzer (CPA), whole blood platelet aggregation (WBPA) studies and thromboelastography (TEG). These have been compared to the traditional *in vivo* assay of skin bleeding time. *Methods.* Single centre, prospective cohort study of patients referred to a tertiary nephrology unit. Patients with both acute and chronic renal impairment were recruited. Laboratory parameters analysed included full blood count, serum creatinine and urea, calculated GFR (Cockcroft formulae), APTT, PT, fibrinogen, SBT (Simplate II), WBPA, PFA-100, TEG and CPA. If patients were on haemodialysis, blood samples were obtained via tunneled vascaths with heparin removed, or via arterio-venous fistulae pre-dialysis. *Results.* This study included 42 patients: 9 with CRF (GFR<30 mL/min) not receiving dialysis; 23 CRF on dialysis; 7 patients presented in acute renal failure; 3 patients assessed had normal renal function but with nephrotic syndrome and presented prior to renal biopsy. 22 patients were on low-dose aspirin and 4 patients were on clopidogrel without significant effect on SBT. There was a poor correlation between calculated glomerular filtration rate (GFR) and SBT (r2=0.1564) and no correlation with serum creatinine or urea. Of the 42 patients 30 patients had SBT >7minutes, 26 patients had SBT >8minutes, 22 patients had SBT >9minutes, and 10 had SBT 15 minutes or greater. Overall no other measure of platelet function predicted for abnormal SBT (see Table 1). In 12 patients with normal SBT, PFA-100 WBPA and CPA assays were abnormal in 7of 12, 5 of 11, and 5 of 7 respectively. Of 10 patients with SBT 15 mins or greater, 2 had normal WBPA, 4 had normal PFA-100 results whilst none of 5 patients tested had normal CPA readings. Overall 19 patients had abnormal TEG tracings, but these were

prothrombotic findings (high MA and increased G parameter). 6 patients underwent renal biopsies with one bleeding complication. This patient had a normal SBT and CPA at the time. *Conclusions.* In this pilot study we found that prolonged SBT were not predicted by serum creatinine or calculated GFR. Within the limitations of this study, an alternative *in vitro* test to replicate the SBT has not been identified.

Table 1. Comparison of assays.

SBT		PFA-100		WBPA		CPA		TEG
Time	n	Not done	Abnormal	Pt on clopidogrel	Abnormal	Not done	Abnormal	Abnorm
<7min	12	1	3/11	0	7/12	5	5/7	6/12
≥7min	30	1	17/29	4	24/26	9	15/21	12/30
<8min	16	1	6/15	1	10/15	5	8/11	8/16
≥8min	26	1	12/25	3	21/23	9	12/17	10/26
<9min	20	2	9/18	1	14/19	6	10/14	9/20
≥9min	22	0	11/22	3	17/19	8	10/14	10/22
≥15min	10	0	6/10	0	8/10	5	5/5	4/10

0425

ANALYSIS OF CLINICAL AND BIOLOGICAL FACTORS ASSOCIATED TO EXCESSIVE BLEEDING IN CARDIAC SURGERY

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Background. Bleeding is the most frequent and important complication associated to cardiopulmonary bypass (CPB) in cardiac surgery. The knowledge of physiopathological aspects related to the own CPB and the use of certain measures (less deep hypotherm, drugs like aprotinin, and better control of the intraoperative anticoagulation, among others) have significantly reduced this hemorrhagic risk. Excessive postsurgery bleeding (EPSB) takes place when the hemorrhage volume is superior to 1 liter in the first 24 hours after surgery. *Aims.* We have analysed what clinical and biological factors are associated to EPSB after cardiac surgery with CPB. *Methods.* We studied 26 patients undergoing cardiac surgery with CPB (15 men and 11 women; median age 67 years, range 40-85), in whom tranexamic acid was not administered during the intervention. Twelve coronary artery bypass operations, 10 valve replacements, and 4 mixed surgeries, were included. Those patients with EPSB were grouped as opposed to those who did not have EPSB, and differences between both groups in relation to physical factors (corporal temperature, haemodynamic indexes), biochemical (BUN, creatinine, CK-MB, CK-NAC, lactic acid, soluble TNF receptor, interleukin-6, complement system and leptin included) and hemogram findings, hemostatic parameters, transfusional requirements and used drugs, were analysed. Data were recorded at four moments: preoperative, arrival at the intensive care unit, after 4 hours of arrival and after 24 hours. The different used statistical tests are explained. *Results.* EPSB was observed in 13 patients (50%). In the preoperative moment, there were no differences between both groups, except for a lower plasma concentration of PAI-1 in the group of patients who showed EPSB. In the moment of arrival at the intensive care unit, those patients who made EPSB presented lower levels of C1q, C1 inhibitor, C7, Factor B of the complement, PAI-1, PT, and leptin, and a lower corporal temperature. After 4 hours of arrival, the patients with EPSB presented lower levels of C1q, C1 inhibitor, C3, C7, Factor B, leptin, PT and fibrinogen. Finally, after 24 hours of arrival at the intensive care unit, the values of C1q, C4 and leptin, were significantly lower in the EPSB-group. We did not find differences in the following factors and parameters: lactic acid, interleukin-6, soluble TNF receptor, APTT, antithrombin, d-dimer, tPA, BUN, creatinine, leukocytes, platelets, CK-NAC and CK-MB, administered dose of dobutamine and noradrenaline, and haemodynamic indexes (cardiac index and systemic vascular resistance index). *Conclusions.* In our experience, several biochemical and hemostatic parameters could serve as predicting factors of EPSB in patients undergoing cardiac surgery. Specifically, some factors of the complement system and leptin (obesity-related protein) seem to play an important role. Our work supports that the activation of the complement system caused by the CPB, could play an important role in the postsurgery hemorrhage.

0426

SUBJECTIVE TRAINING EFFECTS ON ADULT PATIENTS WITH HAEMOPHILIA ATTENDING A SPORTS THERAPY PROGRAMME

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Background. Only since some years sport activities have been recommended for haemophilia patients. Still now the importance of sports therapy as an integral element in haemophilia treatment has not yet been widely recognized. In the frame of the Haemophilia & Exercise Project (HEP) the success of a two years sports specific therapy was evaluated objectively in terms of isometric muscular strength and proprioception and subjectively in terms of the WOMAC questionnaire and the orthopaedic joint score. Subjectively perceived training effects were tested with the a newly developed sport-specific questionnaire (HEP-Test). In addition quality of life was tested with the SF-36 and the haemophilia-specific quality of life questionnaire (Haem-A-QoL). *Aims.* Assessment of subjective training effects of a sports therapy programme for adult patients with haemophilia in terms of bodily condition and quality of life. *Methods.* Based on the contents of the training programme a sport-specific questionnaire (HEP-Test) was developed consisting of 33 items pertaining to 6 dimensions (physical status, mobility, strength & coordination, endurance, body perception, general questions). HEP-Test was pilot tested in 23 German adult haemophilia patients and tested for it's feasibility in terms of acceptance, comprehensibility and relevance. Data were psychometrically analysed in terms of reliability and validity (criterion, convergent, discriminant). Correlation of the HEP-Test with subjective and objective measures were performed. *Results.* From the 23 enrolled patients 87% were severely affected by haemophilia. In 8.7% inhibitors occurred and half of the patients received prophylactic treatment (52.2%). 47.8% of the patients reported target joints. Viral infections were found in 65.2% of the patients (hepatitis C) and in 21.7% for HIV. Concerning the newly developed HEP-Test the mean completion time was 13 minutes; the questionnaire was well accepted and patients found it related to physical activities. Feasibility testing led to the omission of 9 items and suggestions for rewording of some items were given by patients. Psychometric testing revealed excellent characteristics for reliability (Cronbach's α ranging from .82-.90). Validity testing showed high correlation between scales of HEP-Test, SF-36 and WOMAC. Acceptable to high correlation were found with the orthopaedic joint score and the isometric muscular strength test. Discriminant validity testing revealed significant differences for clinical subgroups. *Conclusions.* HEP-Test is a short questionnaire assessing subjective training effects. HEP-Test was well-accepted by patients and showed quite satisfactory psychometric characteristics. Subjective training effects can be measured with the HEP-Test and should be combined with objective assessments in order to reveal aspects, which can not be measured objectively such as body perception.

0427

THE USE OF FEIBA AND NOVOSEVEN FOR TREATMENT OF BLEEDING EPISODES IN PATIENTS WITH HEMOPHILIA A AND FACTOR VIII INHIBITOR: A SINGLE CENTRE EXPERIENCE

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Background. Factor VIII replacement is impossible in hemophiliacs with high titre inhibitor, so FEIBA© and NovoSeven® are the main possibility in treatment of bleeding. *Aims.* Evaluate efficacy and consumption of the products in treatment of bleeding episode in hemophiliacs with factor VIII inhibitor. *Methods.* We used data accumulated in our hemophilia centre in the course of 6 years between 2000 and 2005. Five hemophiliacs with factor VIII inhibitor were treated on demand with FEIBA© (dose 42-79 U/kg 8-12 h) or with NovoSeven® (dose 80-210 µg/kg à 2 h). For efficacy we used evaluation criteria: excellent: bleeding stops within 8 hours from start of treatment, efficient: bleeding stops more than 8 hours following start of treatment, partially efficient: bleeding stops but recurring within 48 hours following stop of bleeding, inefficient: no stop of bleeding after 48 hours of treatment or need for another treatment. *Results.* Patients had 124 bleeding episodes, included 99 spontaneous bleeding episodes (88 hemarthroses, 6 muscle bleedings and 5 other sites bleedings) and 17 traumatic bleedings (9 hemarthroses, one muscle bleeding, 2 other sites bleedings and 5 multiple sites

bleedings) and 8 re-bleeding episodes. Bleeding episodes were treated mostly with NovoSeven® (78) and with FEIBA© (45). We evaluated all episodes (except the re-bleeding episodes) treated with NovoSeven® (71) or with FEIBA© (44). Median total dose per episode was 352 µg/kg and 190 U/kg, dose per infusion was 112 µg/kg and 60 U/kg. In episodes with re-bleeding treated with NovoSeven® (6) median total dose per episode was 382 µg/kg, dose per infusion was 110 µg/kg. Using FEIBA© one episode with re-bleeding had occurred total dose 93 U/kg, 47 U/kg per infusion (dosage was lower than average). The efficacy of NovoSeven® and FEIBA© was excellent in 70% and 47,7% of the episodes, efficient in 21,4% and 47,7%, partially efficient in 8,6% and 2,3%, inefficient in 0 and 2,3%. Separately we evaluated spontaneous hemartroses. NovoSeven® was used in 54 episodes and FEIBA© in 34 episodes. Median total dose per episode was 352 µg/kg and 187 U/kg, dose per infusion was 112 µg/kg and 60 U/kg. In episodes with re-bleeding treated with NovoSeven® (5) median total dose per episode was 368 µg/kg, dose per infusion was 112 µg/kg. Using FEIBA© only one episodes was with re-bleeding it had been mentioned above. The efficacy of NovoSeven® and FEIBA© was excellent in 70,4% and 47,1% of the episodes, efficient in 20,4% and 50%, partially efficient in 9,2% and 2,9%. Median (mean) interval between start of bleeding and start of treatment in spontaneous hemartroses treated with NovoSeven® without re-bleeding was 2,4 h (3,9 h), with re-bleeding was 2,5 (2,7 h). *Conclusion.* In our experience treatment with NovoSeven® stopped bleeding earlier than with FEIBA©, however about 9% of the episodes were with re-bleeding although the dosage used in these episodes and intervals to start of treatment were on the same level as in episodes without re-bleeding. The question is why any bleedings treated in the same way were recurring and the other not.

0428

ORTHOPAEDIC STATUS OF PERSONS WITH HAEMOPHILIA IN A DEVELOPING COUNTRY

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Background. The medical approach of haemophilia, prototype of rare diseases, can be considered a hallmark for the quality of sanitary system in a country. As joints are target sites of bleeding, the orthopaedic status of persons with haemophilia (PWH) is accepted as reliable clinical reflection of diagnostic and therapeutic performances in this disease. *Aims.* We sought to perform a cross-sectional analysis of joint status in terms of physical and functional assessment of haemophiliacs. *Methods.* The study was conducted on 93 patients (77 with haemophilia A and 16 with haemophilia B) consecutively enrolled: 31.18% children and 68.81% adolescents and young adults; 92.47% with a rest-activity <1%. Number of total bleeds and joint bleeding events/ patient/ year, number of patients with target joints and number of target joints/patient, clinical score/joint, global clinical joint score and motor performance as well were analysed. For arthropathy assessment Petrini scale in children and Gilbert scale in adults were used. *Results.* 15.65 bleeds /patient/ year and 11.44 joint bleeds/patient/ year in children, vs. 25.34 and 20.28 respectively in adults were found. Only 27.5% of children, and 9.37% of adults were spared of joint bleeding. More than 50% of adolescents had target joints, 31.17% of them with more than one affected joint. The global joint score was 22.96±21.11 in children and 38.38±20.79 in adults; 23% of patients presented chronic pain, and 75.86% vs. 100% (children vs. adults) live with the burden of functional deficit. *Conclusions.* The deleterious impact of inadequate substitution in haemophilia is evident, not only on joint status, but on quality of life as well. This situation imposes an urgent improvement of the therapy, a costly action, but certainly a cost-efficient one from the point of view of medical economics.

0429

SERUM VASCULAR ENDOTHELIAL GROWTH FACTOR AND THROMBIN-ACTIVATABLE FIBRINOLYSIS INHIBITOR ANTIGEN IN CHILDREN WITH DISSEMINATED INTRAVASCULAR COAGULOPATHY

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Background. Disseminated intravascular coagulopathy (DIC) is a syndrome characterized by systemic intravascular activation of coagulation resulting in depletion of platelets and coagulation factors. Hypercoagulability and hyperfibrinolysis were both thought to be important mechanisms of DIC. Vascular endothelial growth factor (VEGF) is one of the potent angiogenic polypeptides produced by multiple tissues. High amount of VEGF is stored within circulating platelets and is subsequently released during platelet aggregation. When chronic stimulation of VEGF continues vascular hyperpermeability and thrombosis may be induced. We speculated that extremely high value of VEGF in serum of the patients with DIC might be caused via VEGF release in activated platelets. Thrombin-activatable fibrinolysis inhibitor (TAFI) is considered as a modulator of fibrinolysis and therefore it might play an important role in the pathogenesis of DIC. We planned to evaluate the predictive value of serum VEGF and TAFI for the determination of DIC. *Aim.* To evaluate clinical and laboratory findings of 40 consecutive children in a single center, diagnosed as DIC according to ISTH criteria and compare serum VEGF, TAFI levels of these patients with 40 healthy objects to clarify their roles in the pathogenesis of DIC. *Methods.* Forty patients who experienced DIC in our department (Pediatric Hematology Unit of Hacettepe University, Ankara) between December 2003- May 2005 were examined. At the time of diagnosis hemostatic datas of patients with DIC were noted, serum sample of patients with DIC was collected and stored at -80 0 C. *Results.* The underlying diseases of the patients were congenital heart disease (7 patients), chronic renal failure (5 patients), malignancy (3 patients), metabolic disease (3 patients) and collagen tissue disease (2 patients) Twenty four patients had infection, which 17 of them were documented. Mean acute quantitative CRP level was 6, 0±6, 2. At the time of diagnosis median WBC count was 7050/mm^3 (600-154000), platelet count was 70.000/mm^3 (3000-624000) and hemoglobin level was 9,5 gr/dl (5,8-16,3). Low level of protein C and S levels were detected in 13 (32.5%) and 9 (22.5%) patients respectively. Fibrinogen levels were decreased only in six of patients. Majority of patients (37 (92.5%)) had prolonged prothrombin time over 6 seconds. D-dimer levels over 2 g/dL in were detected in 36 (90%) patients. No significant difference were observed in the VEGF levels between study group (320,25±327,33 pq/ml) and healthy controls (514,51±679,19) (p=0.603). There were significant difference in serum TAFI Ag levels between control group (88,9±16,9%) and in patients with DIC (82,3±14,3%) (p=0,007). *Conclusions.* We could not show a correlation between the platelet count and VEGF levels. High value of VEGF in serum of the patients may be overt after the disease progression and serial analysis might be helpful. On the other hand TAFI Ag levels were significantly low in patients with DIC. As it has been suggested that TAFI Ag is mainly under genetic control, further combined approach measuring TAFI levels and TAFI gene polymorphism will be needed.

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Dendritic cells and cellular immunotherapy

0430

EXPRESSION AND REGULATION OF ENDOTHELIAL PROTEIN C RECEPTOR IN MONOCYTE-DERIVED DENDRITIC CELLS

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Background. Endothelial protein C receptor (EPCR) is a transmembrane protein, homologous to MHC class-I molecules, that enhances the rate of protein C activation on endothelial cells. It is reported that EPCR mediates the anti-apoptotic activity of activated protein C on endothelial cells. EPCR was identified also in polymorphonuclear leucocytes and in monocytes. We previously showed by immunohistochemistry that dendritic-like cells in the normal gut mucosa express EPCR. Aims of the study. 1. To characterize phenotypically the gut mucosa EPCR+ dendritic-like cell. 2.To study, in a model of dendritic cell generated *in vitro*, the expression of EPCR and its modulation. *Methods.* EPCR was identified by immunohistochemistry, immunofluorescence or flow cytometry. Dendritic cells *in vitro* were obtained from CD14+ peripheral blood leukocytes, cultured in the presence of interleukin-4 and GM-CSF (MoDCs). Specific messenger RNA (mRNA) was measured by RT-PCR. *Results.* We confirm that the gut mucosa *dendritic-like cells* have a phenotype characteristic of dendritic cells, namely they express CD80, CD83 and HLA-DR. We could not identify by immunohistochemistry EPCR+ dendritic cells in other tissues, such as lymph node, spleen, tonsil, liver, lung, and skin. EPCR surface expression on MoDCs was monitored by flow cytometry together with expression of the DC markers HLA-DR, CD1a, CD80 and CD83. After 7 days of culture, approximately 25% of immature DCs expressed EPCR on their surface. *De novo* expression of EPCR was not correlated with modulation of apoptosis or cell cycle. Lipopolysaccharide-induced terminal maturation of MoDCs down regulated the surface expression of EPCR by 40% while up regulating the expression of CD83. Incubation of cultured DCs with prostaglandin E2 up regulated EPCR mRNA and protein expression by about 3 fold at 50 hours. Flow cytometry studies were compounded by confocal microscopy, which showed that dendritic cell EPCR has the same membrane distribution pattern as in endothelial cells. *Conclusions.* Contact with bacterial antigens modulates EPCR expression on MoDCs, suggesting EPCR might be involved in antigen recognition or processing.

0431

HIGH AFFINITY CYTOTOXIC T CELLS CANNOT OVERCOME THE INTRINSIC RESISTANCE TO THERAPY OF (LEUKEMIC) PRECURSOR CELLS IN DORMANCY

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Most patients with leukemia treated with chemotherapy show a good initial response to therapy. However, despite multiple courses of treatment even in patients initially developing a complete clinical response late relapses of the same leukemia may occur, suggesting that a fraction of the leukemic precursor cells evades the treatment. The selectivity of chemotherapeutic interventions for cells in active cell cycle can explain why treatment with high dose chemotherapy is causing limited harm to normal tissues of the patient. In accordance, we and others have previously demonstrated universal protection of cells in dormancy to conventional chemotherapy. It has been hypothesized that treatment with cytotoxic T lymphocytes expressing a T cell receptor (TCR) with high affinity for the defined antigen (high affinity CTL) would not be hampered by the dormant state of leukemic precursor cells. To analyze this we determined the sensitivity of leukemic cells and normal hematopoietic cells in dormancy and in active cell cycle to cell death induced by high affinity CTL clones recognizing allo-HLA or minor histocompatibility antigens (mHag). We analyzed the sensitivity to antigen-specific killing by T cells of dormant and proliferating normal $CD34^+$ precursor cells and $CD34^+$ CML precursor cells, of normal B cells, T cells and monocytes, and of activated B cells (EBV-LCL) and activated T cells (PHA blasts). In this study we found that all activated, proliferating target cells were very efficiently lysed, resulting in 60-90% lysis already after 4 hours of exposure to the CTL clones (E/T ratios 1/1-5/1). In contrast, target cells in relative dormancy including the non-proliferating CML stem cell fraction, unmanipulated CD34 progenitor cells, and resting T and B cells appeared to be protected from CTL-induced cell death (0-20% lysis). To investigate whether these target cells in dormancy were intrinsically resistant to the effector mechanism used by the T cells or that decreased avidity of the interaction between dormant targets and high affinity effectors was underlying the poor susceptibility, we artificially enhanced the avidity by exogenous loading of the target cells with saturating concentrations of the relevant peptide. This was sufficient to completely restore the sensitivity to levels comparable to activated proliferating target cells, suggesting that reduced avidity of the interaction is playing a significant role in the differential killing of activated and dormant target cells. The differential susceptibility of dormant and activated target cells for T cell recognition may also explain the differential capacity of T cells to cause graft versus host disease (GvHD) in the absence or presence of the *cytokine storm* after alloSCT. We mimicked the cytokine production during GvHD by the addition of interferons. This resulted in a limited, but significant upregulation of the sensitivity of the initially resistant target cell types to recognition by the T cells. In conclusion, we here demonstrate that normal hematopoietic and leukemic cells in dormancy are relatively resistant to cell death induced by high affinity CTL clones. This selective resistance of cells in dormancy is caused by the diminished avidity of the interaction with the CTL.

0432

DIFFERENTIATION TOWARDS LEUKEMIC DENDRITIC CELLS IS HINDERED BY THE PRESENCE OF A FLT-3 INTERNAL TANDEM DUPLICATION IN AML BLASTS

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In the search for new treatment modalities to eradicate minimal residual disease cells (MRD) in acute myeloid leukemia (AML), immunotherapy provides an attractive option. AML blasts show differentiation towards leukemic dendritic cells (DC), providing the unique opportunity to generate DC harbouring the full range of tumour antigens. In a large cohort of AML samples (n=154) AML-DC were generated by two culture methods, i.e. in presence of cytokines GM-CSF, TNF-α, SCF, Flt-3L, IL-3 and IL-4 (n=147) or calcium ionophore (CI) and IL-4 (n=108). Median AML-DC yield, defined by phenotypical DC characteristics, in the cytokine-based cultures was 12% (range:0-70%). Considering cultures yielding ^{3}10% AML-DC successful, 58% (85/147) of cytokine-based cultures were successful and 61% (66/108) of CI cultures. Overall, functional AML-DC generated with either method was possible in 66% (101/154) of patients. Identification of AML blast populations with DC differentiation capacity is important to select patients eligible for immunisation programs. Interestingly, presence of Flt-3 internal tandem duplication (ITD) was strongly correlated with decreased DC differentiation capacity in both culture methods (cytokine-based culture: $p<0.001$; CI-culture: $p=0.03$) suggesting that constitutive activation of tyrosine kinase receptors inhibits differentiation towards DC. In multiparameter regression analysis, powerful predictors for cytokine-based AML-DC culture outcomes were Flt-3 ITD (regression coefficient B:-3.41, $p<0.001$), CD14 (B:3.28, $p<0.001$) and TNFα-RI (B:2.82, $p<0.001$). This regression model predicts 88% of culture outcomes. ROC curves show high sensitivity (95%) and specificity (76%) with an AUC of 0.93 ($p<0.001$). In 25% of unsuccessful cytokine-based cultures, the CI-based culture method provides an alternative. This percentage increases to 56% if Flt-3 ITD+ AML samples are left out, emphasizing Flt-3 ITD+ blasts' inability to differentiate towards leukemic DC. In conclusion, AML-DC cultures are successful in most patients. Selection of patients is well possible based upon the presence of Flt-3 ITD and the expression of CD14 and TNFa-RI. Based on these results, we are currently entering patients in a phase I/II clinical vaccination trial.

0433

THE NOTCH LIGAND DELTA-LIKE1 PROMOTES THE GENERATION OF INTERSTITIAL AND PLASMACYTOID DENDRITIC CELLS FROM HUMAN MYELOID COMMITTED PROGENITOR CELLS

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The Notch ligand Delta-like1 (DL-1) and the ETS-transcription factor PU.1 are key regulators of dendritic cell development. We recently observed that ectopic expression of PU.1 in CD34+ cord blood progenitor cells promotes the development of human conventional CD1a+ DCs (cDC), compromising Langerhans cells and interstitial type DCs.

DL-1 induces CD1a+ DCs from human progenitors at the expense of CD14+/CD11b+ monocytes, and this effect is associated with the upregulation of PU.1. Plasmacytoid DCs (pDC) represent a third DC subset which can be generated from both, granulomonocyte progenitors and lymphoid progenitors. In contrast to cDCs, they lack myeloid markers and CD1a, and are PU.1 low/negative. Their lineage relationship to cDCs remains poorly characterized. We generated myeloid progenitors (MP) by expanding cord blood CD34+ cells with early acting cytokines *in vitro*. Using the OP9 stroma culture system, we here show that DL-1 promotes pDC development from MPs. Notch signaling promoted pDC generation from MPs at the expense of CD11b+ cells. These data suggest that pDCs and cDCs share a common myelomonocytic committed progenitor cell, and that Notch and PU.1, together with additional factors, might induce the lineage specification of pDCs versus cDCs.

0434

VACCINATION WITH WT1 AND PR3-DERIVED PEPTIDES IN PATIENTS WITH AML/MDS AND MUC1-DERIVED PEPTIDES IN PATIENTS WITH MULTIPLE MYELOMA - PRELIMINARY RESULTS

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Background. It has been demonstrated that the Wilms Tumor gene (WT1) is highly expressed in various types of leukaemia. WT1 expression level reflects the extent of minimal residual disease and significantly increases at relapse. Proteinase 3 is an aberrantly expressed myeloid leukaemia protein and T cells with specificity for both, Wt1 and Pr3-derived antigens, have been generated *in vitro* from healthy individuals and cancer patients and lysed myeloid leukaemic blasts. MUC1(CD227) is presented on a considerable amount of multiple myeloma cell lines and plasmacytoma cells, but only some B-cells. Several lines of investigation have provided conclusive evidence that MUC1-derived HLA-class I/II epitopes do represent universal tumor antigens, which are also expressed by malignant plasmacytoma cells and could thus be attacked by MUC1-specific CTLs. *Aims.* We investigate safety and feasibility of a vaccination with WT1 and Pr3 or MUC1-derived peptides in patients suffering from AML or multiple myeloma, respectively. Thereby we asses the induction of tumor-antigen specific T-cells as well as clinical responses. *Methods.* HLA A2.1 positive patients with AML/MDS <30% blasts in the bone marrow biopsy receive 6 injections of WT1 and Pr3-derived peptides, combined with Montanide ISA51 (incomplete Freund´s adjuvants), PADRE and VaxImmune (CpG 7909). Vaccination is given every two weeks. HLA A2.1 positive patients with multiple myeloma Stage I, stable disease or partial remission after chemotherapy receive 6 injections of two different MUC1-derived peptides, VaxImmune and Montanide with or without PADRE. Safety and feasibility as well as clinical course is reassessed every visit. Induction of immune response is assessed by ELISPOT, Cr-release-Assays and FACS-analysis (Tetramer-staining). *Results.* So far, three patients completed our ongoing AML-vaccination protocol; four patients were vaccinated in the myeloma-study. A total of 10 patients will be treated in each trial. Local inflammatory responses at the injection site, such as redness, swelling and pain were observed in all patients (Grade II). In one case, skin necrosis (Grade II) and superinfection occurs. Four out of seven patients developed a systemic reaction including influenza-like symptoms and fever (Grade I-II). One patient suffered from an anaphylactic reaction (GRADE III) after vaccination. Clinical response data have so far been analysed for patients vaccinated with Pr3 and WT1-derived peptides: Peripheral platelet counts of a patient suffering from MDS RAEB-T improved while blasts detected in the bone marrow remained stable. Two out of three patients with refractory AML remained progressive even after six vaccinations. Clinical responses of patients treated with MUC1-derived vaccine as well as immunological analyses of all vaccinated patients are pending and will be presented at the meeting. *Summary / Conclusion.* Vaccination with WT1/ PR1 or MUC1, PADRE, VaxImmune (CpG7909) and Montanide is safe and feasible, even in advanced disease and after multiple previous therapies, including stem cell transplantation. Observed local as well as systemic side effects were predominantly mild to moderate. Overall clinical and immunological response data will be presented.

0435

CANCER-TESTIS ANTIGENS ARE COMMONLY EXPRESSED IN MULTIPLE MYELOMA AND INDUCE SYSTEMIC IMMUNITY FOLLOWING ALLOGENEIC STEM CELL TRANSPLANTATION

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Background. Immunotherapies using cancer-tesis (CT) antigens as targets represent a potentially useful treatment in patients with multiple myeloma (MM) who commonly show recurrent disease following chemotherapy. Furthermore, CT antigens might represent targets for graft-versus-leukemia (GvL) effects following allogeneic stem cell transplantation (alloSCT). *Methods.* We analyzed the expression of 11 CT antigens in bone marrow samples from MM patients (N=107) and healthy donors (N=32). Furthermore, we analyzed 66 MM patients for antibody responses against MAGEA3, SSX2, and NY-ESO-1 in an ELISA assay. Finally, we screened a patient with a humoral responses agsinst NY-ESO-1 for T cells against the same antigen in an ELISPOT assay using overlapping peptides. *Results.* CT antigens were frequently expressed in MM with 56% (MAGEC2), 55% (MAGEA3), 35% (SSX1), 20% (SSX4, SSX5), 16% (SSX2), 15% (BAGE), 7% (NY-ESO-1), and 6% (ADAM2, LIPI) expressing the given CT antigen (see Figure). Importantly, with the exception of SSX4 none of the CT antigens were expressed in healthy bone marrow. Analyzing our patients for IgG antibodies against MAGEA3, SSX2, and NY-ESO-1, we found strong antibody responses against CT antigens in 9 patients who had received alloSCT. Antibody responses against NY-ESO-1 correlated with NY-ESO-1-specific CD4+ and CD8+ T cell responses against peptide NY-ESO-1 51-62 and CD4+ responses against NY-ESO-1 121-140 in one of these patients. These allogeneic immune responses were not detectable in pre-transplant samples and in the patients' stem cell donors indicating that CT antigens might indeed represent natural targets for graft-versus-leukemia effects. *Conclusions.* We show here for the first time that CT antigens induce spontaneous antibody and T cell responses in MM patients who received alloSCT. These immune responses induced by alloSCT could probably be boosted by active CT antigen-specific immunotherapy which might help to achieve long-lasting remissions in patients with MM.

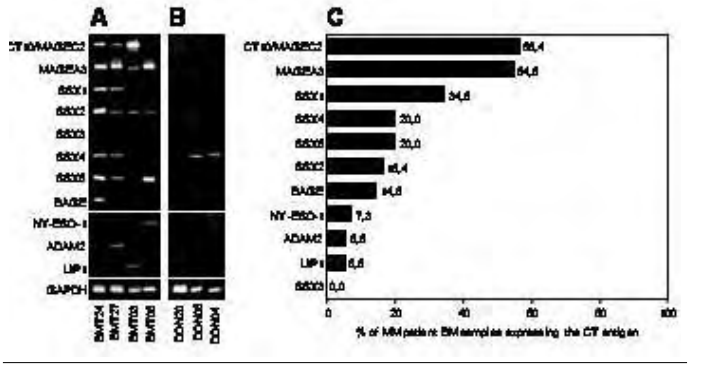

Figure 1. Expression of CT antigens in MM.

0436

PHENOTYPIC CHARACTERIZATION OF PLASMACYTOID DENDRITIC CELLS LINEAGE MATURATION PATHWAY IN NORMAL ADULT BONE MARROW: A FRAME OF REFERENCE FOR UNDERSTANDING DENDRITIC CELL MALIGNANCIES

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Background. A new entity of haematological malignancy has been identified recently as arising from plasmacytoid dendritic cell (pDC) precursors, the maturation pathway of their normal counterpart in bone marrow (BM) being largely unknown. *Aim.* To analyze the immunophenotypic features and their changes associated to pDC maturation in BM samples from normal adults as a frame of reference for better identifying pDC precursor malignancies. *Methods.* A total of 25 BM samples were stained by direct immunofluorescence techniques using a large panel of monoclonal antibodies in 6-color combinations. Data acquisition was performed in a FACSCanto flow cytometer and the FACSDiva software program was used for data analysis. *Results.* In all cases, three pDC maturation stages were clearly identified based on the expression of CD34, HLA-DR, CD123 and CD45. Accordingly, the more immature precursors (stage I), represent-

ed 17%±6% of all pDC and showed a CD34++/HLA-DR+++/CD123++/CD45+ phenotype; intermediate stage pDC (stage II), represented 21%±6% of all pDC and they were CD34+/HLA-DR++/CD123+++/CD45++ and; the more mature pDC (52%±11%) were CD34-/HLA-DR++/CD123+++/CD45++ (stage III). Both HLA class I and class II molecules showed a high expression in stage I pDC, which decreased in the intermediate stage to finally recover and remaining expressed at high levels in the more mature pDC stage. Concerning DC-associated molecules, BM pDC were negative for CD1a, CD1b, CD1c, CD209, CD275 and the TLR4 and TLR9 Toll-like receptors, while CD303 (BDCA2), CD304 (BDCA4) and CD85j appeared in the more immature stage, progressively increasing their levels along the differentiation of pDC; CD4 showed an identical pattern. Expression of other molecules, such as CD11a, CD36, CD38, CD45, CD45RA, CD54, CD62L, CD184 and CD197 was high and quite stable through maturation of pDC; in contrast, expression of CD11c, CD13, CD33, CD64, CD99, CD116, CD117 and CD126 progressively decreased during pDC differentiation, becoming negative in stage III pDC. Interestingly, the CD86 co-stimulatory molecule was detected at high levels in stage I, and dropped dramatically afterwards. In contrast, CD40 was only detected in stage III. CLA was heterogeneously expressed throughout the maturation. *Conclusion.* In summary, we show that at least three maturation stages of pDC are identifiable in normal adult BM, on the basis of their different phenotypic characteristics, this representing a frame of reference for a better identification and understanding of pDC precursor malignancies.

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0437

COMBINED TYROSINE KINASE INHIBITION AND IMMUNOTHERAPY AS A STRATEGY TO IMPROVE OUTCOME AFTER REDUCED INTENSITY ALLOGENEIC STEM CELL TRANSPLANTATION IN CHRONIC MYELOID LEUKAEMIA

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*Background.*The graft-versus-leukaemia effect of allogeneic stem cell transplantation and donor lymphocyte infusions (DLI) is capable of producing long term disease free survival in CML. Despite this the toxicity of the conditioning regimen and risk of graft-versus-host disease (GVHD) make allografting an unattractive therapeutic option in most patients. Recently it has been shown that reduced intensity conditioning (RIC) regimens incorporating alemtuzemab reduce both transplant related mortality and GVHD risk. However such regimens are associated with a high rate of relapse which occurs in most patients within the first year post-transplant. Whilst DLI is a safe and effective salvage therapy after a myeloablative transplant, its use is associated with a significant risk of severe GVHD in patients who relapse early after a RIC transplant. Therefore strategies which permit the effective use of DLI after RIC allografts are required if they are to fulfill their curative potential. *Aim.*We have studied whether the administration of leukaemia specific therapy in the form of the tyrosine kinase inhibitor Imatinib for a limited period after a RIC transplant can postpone the requirement for DLI thereby reducing its toxicity in patients who eventually relapse. *Methods.*Patients with an available sibling donor underwent allogeneic stem cell transplantation using fludarabine 25mg/m^2/day iv (days -7 to -3), Busulphan 8 mg/m^2 orally (days -3, -2) and alemtuzemab 10 mg/day iv (days -7 to -3), Cyclosporin was used as GVHD prophylaxis. Imatinib was commenced on day +35 and continued until one year post transplant at 400mg daily. Minimal residual disease levels were measured by quantitation of BCR:ABL transcript numbers at three monthly intervals. Escalating dose DLI was administered in patients who relapsed after the discontinuation of Imatinib. *Results.* 21 patients with CML in 1st chronic phase were transplanted between March 2002 and September 2005. The median age was 49 (25-57). All patients engrafted promptly and commenced Imatinib on day +35. All tolerated continuous treatment until one year post-transplant apart from 3 in whom it was temporarily discontinued because of gastrointestinal intolerance. The day 100 transplant related mortality was 0%. Only one patient developed acute GVHD (grade 3). All patients achieved mixed T cell chimaerism by day +90. All patients demonstrated a greater than three log reduction in BCR:ABL transcript numbers in the first year post-transplant: 10 achieving molecular remission. After discontinuation of Imatinib DLI was instituted in 11 patients because of disease relapse. 7 patients have completed a course of DLI - 6 of whom have achieved durable molecular remissions. 4 patients developed GVHD in association with DLI. *Summary*. We conclude that the combination of Imatinib and a RIC allograft is remarkably well tolerated in patients with CML and may allow the subsequent delivery of DLI without compromising its ability to produce molecular remission. The use of adjunctive leukaemia specific therapy may prove an effective strategy to optimise the immunotherapeutic potential of DLI and improve outcome after RIC allografts in other diseases in which targeted therapies are available.

0438

CYTOKINE INDUCED KILLER CELLS TRANSDUCED WITH ANTI-CD19 CHIMERIC RECEPTORS CONTAINING 4-1BB HAVE POWERFUL ANTI-LEUKEMIC ACTIVITY

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Background. CIK cells are a population of ex-vivo expanded cells with MHC-unrestricted cytotoxicity against several tumoral targets, except B-lineage Acute Lymphoblastic Leukemia (ALL). We have recently demonstrated that transduction of an anti-CD19-ζ chimeric receptor in CIK cells rendered them efficient killers of CIK-resistant ALL cells. Conceivably, the capacity to proliferate after contact with leukemic cells and to exert prolonged anti-leukemic cytotoxicity after infusion should be important to maximize the likelihood of success of this cell therapy. It was previously shown that incorporation of costimulatory molecules into chimeric receptors markedly enhances target-cell stimulated proliferation and cytotoxicity in T lymphocytes and Natural Killer cells. *Aims.* to identify costimulatory molecules that increase the cytolitic activity and proliferative capacity of anti-CD19-ζ receptor transduced CIK cells. *Methods.* CIK cells were transduced with a RD114-pseudotyped retroviral vector carrying different types of receptors: anti-CD19-ζ, anti-CD19-DAP10, anti-CD19-4-1BB-ζ and anti-CD19-CD28-ζ. A truncated form of the receptor was used as control. The cytotoxic activity of transduced CIK cells against ALL cells was detected by co-colture with the OP-1 B-lineage ALL cell line for 4 hours (short-term cytotoxic assay) or for 6 days on a mesenchimal cell layer (long-term cytotoxic assay). The recovery of ALL cells was then evaluated by flow cytometry. CIK cell proliferation was assessed in cocultures with irradiated OP-1 cells and low-dose IL-2. *Results.* CIK cells were efficiently transduced with the anti-CD19 retroviral vectors (average expression of GFP and chimeric receptor, 55% for all vectors tested; n = 5 each). After 4 hours of incubation, CIK cells expressing anti-CD19-ζ anti-CD19-DAP10, anti-CD19-CD28-ζ and anti-CD19-4-1BB-ζ receptors were all strongly cytotoxic against OP-1 cells (>60% of lysis at E : T ratio 2:1 for all receptors tested). However, the benefits of adding the costimulatory molecules 4-1BB or CD28 to the receptor was evident in long-term assays with low percentages of CIK cells (E:T ratio 0.01:1). In these assays, CIK cells expressing anti-CD19-4-1BB-ζ or anti-CD19-CD28-ζ receptors had more potent cytotoxicity than cells expressing the anti-CD19-ζ receptors: in experiments with 4 donors average cell killing was 92.8% (range, 89.4%-97.6%) 93.5% (87.0%-96.8%), and 13.8% (2.9%-23.6%), respectively (p=0.001). By contrast, addition of DAP10 to the receptor did not improve cytotoxicity: average cell killing 2.3% (2.2%-2.4%). Notably, CIK cells transduced with the anti-CD19-4-1BB-ζ receptor had higher proliferative capacity in cocultures with OP1 and low dose IL-2. The average fold increase after 2 weeks of colture was 2.6 (range, 2.4-3.0) for these cells while expansion of cells transduced with either anti-CD19-ζ or anti-CD19-CD28-ζ was 1.4 (range, 1.3 - 1.5) and 1.7 (1.2 - 2.7), respectively (p=0.01). *Conclusions.* expression of anti-CD19-4-1BB-ζ chimeric receptors in CIK cells confers powerful and specific cytotoxic activity against ALL cells, and induces their proliferation. We suggest that anti-CD19-4-1BB-ζ expressing CIK cells may be an attractive strategy for cell therapy of ALL.

0439

INTRACELLULAR EXPRESSION ANGIOTENSIN-CONVERTING ENZYME (ACE, CD143) BY LEUKEMIC DENRITIC CELLS IN ACUTE MYELOID LEUKEMIA (AML) PATIENTS

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Background. Dendritic cells (DC) play a key role in the induction of adoptive immune response because they are efficient in antigen presen-

tation and costimulation of naïve lymphocytes. AML blast cells can be induced to differentiate into leukemic dendritic cells (LDC). LDC are quite similar to dendritic cells derived from monocytes of healthy donors (DC) by the expression profile of integrins and co-stimulatory molecules. The only difference between LDC and DC is the absence of surface ACE expression on LDC in contrast to high level of ACE on DC. *Aim.* We propose that the absence of surface ACE expression in LDC is due to the block of normal ACE transport to the cell surface. To confirm this hypothesis we quantified the level of intracellular and surface ACE in LDC and DC. *Methods.* Blood samples were collected from 10 AML patients at diagnosis before induction chemotherapy and 5 healthy donors (control group). Mononuclear cells were isolated using gradient centrifugation with Ficoll-Paque and were differentiated into dendritic cells by culturing with 180 ng/mL calcium ionophore for 4 days. DC were stained for surface and intracellular ACE using two mAbs to ACE - 1D8 for unfolded ACE (mainly located intracellular) and 9B9 for both native (surface) and unfolded ACE and further analyzed by flow cytometry. *Results.* LDC derived from AML blasts did not express surface ACE 1.5±0.9% of positive cells - clone 1D8 and 2,4±2% - clone 9B9. However, LDC contained a large amount of intracellular ACE: 67±16% (clone 1D8), 81,3±9% (clone 9B9). In contrast, surface ACE expression was revealed in 46,5 ±5,4% of donors DC (with mAb 9B9). DC derived from monocytes of healthy donors had lower intracellular ACE 10,3±9% of cells (with mAb 1D8), and 34,8±31,5% (with mAb 9B9). The proportion of intracellular ACE expressing LDC was significantly higher than in DC (p<0,0001 and p<0,001 for clones 1D8 and 9B9, respectively). The proportion of surface ACE positive LDC was significantly lower than surface ACE positive DC (p<0,0001 for clone 9B9). *Conclusion.* The data demonstrate the block of ACE transport to the cell surface of LDC and therefore, provide another evidence of the distorted differentiation capacity of AML blasts.

0440

AUTOLOGOUS IMMUNOTHERAPY WITH CYTOKINE INDUCED KILLER CELLS FOR HEMATOPOIETIC AND SOLID TUMORS

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Background. Immunotherapy represents a promising treatment strategy for many types of cancer. This approach is hampered by the difficulty in generating sufficient number of cytotoxic cells especially in patients heavily treated. CIK cells are a novel population of immune effector cells CD3+CD56+ with high proliferation rate and potent antitumor activity against a variety of tumor cells targets through a perforin based mechanism mediated by NKG2D. We started a pilot clinical trial in patients with refractory lymphoma and metastatic solid tumors according to GMP guidelines that is currently ongoing. The aim of this study is to assess the toxicity of this regimen, and to evaluate its ability to exert antitumor effect. *Methods.* CIK cells were generated from PBMNC and incubated in LifeCell culture bags in the presence of IFN-γ followed by IL-1b, OKT3 and IL-2. Expansion was assessed between day 21 and 28 and flow cytometric analysis was performed every week. Patients were monitored before and after treatment. *Results.* We enrolled 11 patients: 6 advanced lymphomas, 4 metastatic kidney carcinoma (RCC) and 1 hepatocellular carcinoma (HC). The median number of transferred cells per patient was 19×10^9 ($6\text{-}37\times10^9$) and the absolute number of CD3+CD56+ cells infused ranged from 1 to 16×10^9 (median value 5×10^7/Kg). Patients affected by solid tumors received in association low doses of rhIL-2 or a-interferon. Protocol adherence was excellent and the toxicity profile was favourable. Only 2 patients developed low-grade fever during the first cycle of infusions (5%), recovered without antibiotic treatment. After CIK cells infusion, in patient's peripheral blood the absolute median count of PBLs, CD3+, CD8+ and CD3+CD56+ cells significantly increased with a p-value of 0.034, 0.025, 0.034 and 0.038, respectively. Clinical outcome appeared promising. 2 of the 7 evaluable patients achieved complete response: 1 RCC and 1 HC and 2 patients had stabilization of disease (1 NHL and 1 RCC). At the last follow-up they are still alive at 26, 14, 19 and 9 months respectively, after the start of therapy. *Conclusions.* These preliminary data showed that adoptive immunotherapy with CIK cells is a safe therapy with some suggestion of efficacy that significantly enhances immune functions increasing absolute numbers of effector cells without side effects. If confirmed in larger scale studies, these promising results may have a favourable impact on conventional treatment strategy of malignancies.

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0441

WHOLE CML LYSATE-PULSED DENDRITIC CELLS INDUCE SPECIFIC T CELL RESPONSES TOWARDS CML PROGENITOR CELLS INDICATING THEIR POTENCY FOR APPLICATION IN ACTIVE SPECIFIC IMMUNOTHERAPY

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Background. Treatment of chronic myeloid leukemia (CML) with Glivec results in high rates of cytogenetic responses. However, in many patients, molecular techniques still detect minimal residual disease (MRD). Dendritic cell (DC) based immunotherapy might be an interesting approach to eradicate MRD. We have previously shown that intradermal vaccination with *in vitro* generated autologous CML-DC in patients in late chronic phase CML induced an anti-leukemia immune response demonstrated as strong delayed type of hypersensitivity (DTH). However, CML-derived DC harbor functional defects with respect to migratory capacity and antigen presentation. Similar to CML-derived DC, whole CML lysates loaded onto monocyte derived DC might enable the presentation of both known (e.g. BCR-ABL and WT-1) and unknown tumor-associated antigens. *Aim.* In this study we evaluated the potency of normal monocyte-derived DC (MoDC) pulsed with whole CML lysate to stimulate immune responses towards CML progenitor cells in order to develop DC-based immunotherapy. *Methods.* Donor MoDC were generated in serum-free medium using GM-CSF and IL-4. Part of these DC was pulsed with whole-CML lysate for 2h. (1 DC:1 CML cell) where after all DC were matured with a standard mixture of inflammatory cytokines TNF-α, IL-1b, IL-6 and prostaglandin E2. During 5-6 weeks, donor CD8+ T cells were weekly stimulated with irradiated MoDC or lysate-pulsed MoDC (LP-MoDC) in the presence of IL-2 and IL-7. *Results.* In three separate experiments, T cell subpopulation analysis showed a preferential outgrowth of effector-memory cells in both situations (from 10-15% at start of co-culture culture up to 60-95% after 5-6 weeks); LP-MoDC stimulation resulted in highest percentages of effector T cells, median 5.3% versus 1.4% in non-lysate cultures. At the end of co-culture, the number of IFN-g-producing T cells was 2.4 fold higher when cells were stimulated with LP-MoDC. TCR-Vb analysis revealed the presence of at least one additional T cell clone per experiment as compared to non-lysate MoDC stimulation. These clones represented approximately 16% (median value) of the CD8+ T cells. Furthermore, WT-1 specific T cells were detected within LP-MoDC-stimulated T cells (median 0.21%) indicating leukemia-associated antigen specificity. Moreover, LP-MoDC-stimulated T cells killed more CML CD34+progenitor cells (corresponding to the origin of the lysate) than MoDC-stimulated T cells (median apoptosis/necrosis 63% vs. 38% at E:T=10:1 as detected by a flow-cytometric cytotoxicity assay, respectively). In 2/3 cases, it was shown that induction of apoptosis/necrosis in CML progenitors by LP-MoDC-stimulated T cells was MHC class I-restricted. *Conclusions.* Our data show the potency of CML-lysate-pulsed DC to induce anti-tumor responses and support their utilization in DC-based vaccination strategies to eradicate MRD persisting after Glivec treatment in CML.

0442

ROLE OF COMMON Γ-CHAIN CYTOKINES IN THE MODULATION OF PROLIFERATING CAPACITY AND ON DISTRIBUTION OF T MEMORY AND EFFECTOR CELLS OF *EX VIVO* GENERATED HUMAN DONOR-DERIVED ANTI-LEUKAEMIA CTL LINES

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Background. The success of adoptive transfer of anti-tumor CTL lines depends on the number of infused cells and on their long-term survival and proliferating capacity *in vivo*. We have demonstrated the possibility of generating and expanding *in vitro* a large number of long term CTL lines, directed towards different types of acute leukemia blasts (LB), to be employed in approaches of adoptive immunotherapy aimed at preventing/treating leukemia relapse after allogeneic HSCT. Anti-leukemia CTL lines were generated from CD8-enriched donor T lymphocytes stimulated with donor-derived immature DC pulsed with patients leukemia blasts (LB), in the presence of IL-12 and IL-7 during the primary cultures. Cultures were expanded by means of three rounds of leukemia-specific stimulation and one round of antigen-independent expansion supported by low doses of IL-2. It has been recently demonstrated, that common γ-chain cytokines, in particular IL-7 and IL-15 can promote T-cell proliferation and are required for the development or maintenance

of memory T cells. *Aims.* Based on these data, in this study we evaluated the possibility of modulating the anti-leukemia response, in terms of specificity and phenotypic characteristics of CTLs, using common γ-chain cytokines (IL-2, IL-7 and IL-15) at different phases of the *in vitro* induction and expansion of anti-leukaemia CTL lines. *Methods.* In particular, anti-leukaemia CTL lines supported with the different cytokines were compared for i) levels of the *in vitro* proliferation and cytotoxic activity against both patient LB or non malignant cells, and ii) the distribution of T memory and effector subsets. *Results.* We found that, even though sizeable levels of anti-leukaemia T-cell response can be obtained in all cultures, the use of different cytokines during the various phases of the induction of antileukemia CTL response allows us to modulate not only the expansion rate of anti-leukaemia CTLs and their leukaemia-directed cytotoxicity, but also the percentage and the absolute number of T memory and effector cells, without loss of specificity. *Conclusions.* In particular, we demonstrated the crucial role of IL-15, in increasing T central memory (TCM) cells, potentially able to display long-term survival and capacity to *in vivo* proliferate in the presence of limited amount of LB. Further experiments are in progress to confirm this phenomenon and to evaluate precisely the role of IL-7 in the maintenance of TCM cells during the expansion of anti-leukaemia CTLs.

0443

IMMUNOGENIC PEPTIDE DERIVED FROM NM23-H2 AS A POTENTIAL TARGET FOR LEUKEMIA-SPECIFIC IMMUNOTHERAPY

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Background. Human leukemia cells bear HLA-restricted, tumor associated antigens (TAA) recognized by allogeneic CD8+ cytotoxic T-lymphocytes (CTL). The aim of this analysis was to search for new TAAs on leukemic cells by establishing a T-cell line (CTL) from a patient with CML, and identifying the corresponding immunogenic peptides by cDNA expression cloning. *Methods.* Irradiated leukemic cells of a patient with CML were used in an *in vitro* stimulation of PBMC derived from his HLA-identical related donor, resulting in CTL expansion in excess of 2 x 108 cells. An IFN-γ Elispot assay was used to test the specificity of the CTL against a panel of normal and malignant target cells. HLA-restriction was determined by blocking experiments using mAb directed against commons determinants of HLA class I (W6.32, B1.23.2, SRF8-B6, GAP-A3). Subsequently, cDNA libraries from the leukemic cells were established in the eukaryotic expression vector pcDNA3.1/V5HISTOPO and picked into multiple pools of 100 colonies each. 293T cells were then co-transfected with the colony-pool cDNA together with the appropriate HLA-cDNA and tested in an IFN-γ Elispot assay 48h later. Positive cDNA pools were then further subdivided into pools of 10 colonies, and finally to single colonies, re-analysing at each stage to identify the reactive cDNA clones. Positive clones were then sequenced on both strands. The entire ORF of Nm23-H2 was amplified by PCR, cloned into an expression vector and transfected together with HLA-A32 cDNA into 293T cells to confirm reactivity in the Elispot assay. The immunogenicity of synthetic peptides was determined by direct loading of HLA-A32 expressing APCs. *Results.* The CML-specific CTL recognized leukemic PBMC, CD14+ and CD34+ subpopulations as well as EBV-LCL (bcr/abl+) in a HLA class I restricted manner, without recognising CD4+ and CD8+ subpopulations or bcr/abl negative PHA blasts. Further analysis revealed restricted recognition involving HLA-A32, HLA-B44 and HLA-C03. After testing of one-thousand '100' and four hundred '10' cDNA pools, a total of three single cDNA clones were recognized by the CTL in a HLA-A32 restricted fashion. All three cDNAs were found to be identical to Nm23-H2 (an NDPkinase and transcriptional regulator of cMyc) and to contain no mutations. An independently cloned Nm23-H2 sequence co-transfected with HLA-32 was specifically recognized by the CTL. Although the identification of immunogenic peptides within this sequence was complicated by uncertainty concerning the anchors of HLA-32, one of three candidate peptides tested (Nm2370-78) was found to be recognised specifically with relatively high avidity in peptide-pulsed target cells (50% maximal specific recognition at 1 nM). *Conclusion.* Peptide Nm2370-78 derived from Nm23-H2 is immunogenic and a potential target for specific immunotherapy of CML patients.

0444

CROSS-COSTIMULATION BY DONOR ANTIGEN PRESENTING CELLS PLAYS A ROLE IN ACUTE XENOGENEIC GRAFT-VERSUS-HOST DISEASE

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Background. Graft-versus-host disease (GvHD) limits the efficacy of allogeneic cellular immunotherapy for the cure of human disease. In mouse models, acute GvHD appear to be initiated by the encounter of donor T cells with host antigen presenting cells (APC). Cross-presentation of host antigens by donor APC arising *de novo* from the hemopoietic cell graft also participates to GvHD. *Aim.* To verify if the adoptive transfer of donor APC along with T cells plays a role in acute GvHD. *Methods.* Adoptive transfer of human T cells in conditioned non-obese diabetic (NOD)/severe combined immunodeficient (scid) and evaluation of the requirements for acute xenogeneic GvHD. *Results. In vitro*, human blood mononuclear cells (PBMC) proliferate in response to dendritic cells (DC) derived from NOD/scid mice. Proliferation depends on the presence of human APC. Poising costimulatory properties of human APC by chemical treatment inhibits proliferation, while neither blocking MHC class II-restricted antigen presentation with anti-human antibodies nor interfering with antigen processing by chloroquine does. The intraperitoneal transfer of human PBMC into sub-lethally irradiated NOD/scid mice causes acute xenogeneic GvHD in a dose dependent manner. After NK cell inactivation, the intravenous route is also effective. In the latter setting, depletion of human APC from PBMC significantly reduces the incidence and the severity of xenogeneic GvHD. *Conclusions.* The adoptive transfer of donor APC along with T cells exacerbates acute GvHD possibly through costimulation provided in trans (cross-costimulation). This has important implications for the designing of novel therapeutic strategies.

0445

DEVELOPMENT AND CHARACTERISATION OF GMP-GRADE CYTOMEGALOVIRUS PP65-SPECIFIC CD8+ AND CD4+ T-CELL LINES FOR ADOPTIVE TRANSFER

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Background. Reactivation of cytomegalovirus (CMV) remains a major cause of morbidity and mortality during the period of immune deficiency following allogeneic stem cell transplantation. Antiviral pharmacotherapy is not satisfactory due to significant toxicity and moderate efficacy. It has been shown that adoptive transfer of donor-derived CMV-specific T-cells may be an effective strategy to control established CMV infection. For a persistent function *in vivo* the transfer of both virus-specific CD8+ and CD4+ T-cells is essential. *Aims.* In this study we developed a protocol for the generation of CMV pp65-specific CD8+ and CD4+ T-cell lines for adoptive transfer. The isolation and culture conditions were optimised resulting in a suitable approach for clinical implementation which is fully compliable with Good Manufacturing Practice (GMP) conditions. *Methods.* PBMCs from five CMV seropositive donors were stimulated with different concentrations (6,6-66 μg/mL) of recombinant CMV pp65 protein (Miltenyi Biotec) and/or HLA-A*0201/HLA-B*0702 restricted immunodominant pp65 peptides (NLV/TPR). Peptides used were clinical grade and recombinant protein was γ-irradiated (50 kGy, '80 C°) to eliminate possible microbiological contamination. IFNγ producing cells were enriched using the IFN_ secretion assay (Miltenyi Biotec) at day 1 after stimulation, and cultured with autologous feeders (10x) and low or high dose of IL-2 (10 or 50 IU IL-2/mL). At day 7-11 cells were harvested and cryopreserved. Cell lines were analysed at different time points for staining by peptide-MHC tetramer (NLV-A2/TPR-B7) and phenotypic markers. In addition, pp65-specificity was evaluated by intracellular IFNγ staining after restimulation with a pp65 protein-spanning pool of 15-mer peptides. CMV-specific lysis was tested in a 51-chromium release assay on pp65-transduced target cells. RESULTS ' Enrichment of IFNγ producing cells after pp65 protein stimulation resulted in pp65-specific cell lines consisting of both CD8+ (median 28%, range 20-74%) and CD4+ T-cells (median 48%, range 12-79%). The CD8+ compartment contained immunodominant tetramer staining cells (median 60%, range 5-75%). The majority of both CD8+ and CD4+ T-cells produced IFN_ on restimulation with the pp65 peptide-pool and cell lines showed CMV-specific lysis of target cells. The phenotype of pp65-specific T-cells was predominant CD28+/CD45RO+ and CD45RA-/CCR7-/CD62L-, although CCR7 and CD62L were transiently expressed at day 4 and 7 after stimulation. Addition of higher concentrations of protein during the initial stimulation had a negative effect on enrichment

probably due to non-specific stimulation of cells. Addition of immunodominant pp65 peptides resulted in stronger stimulation and proliferation of epitope-specific CD8+ T-cells, although isolation efficiency was not increased. Except for the enhancement of proliferation, no effect of high dose compared to low dose IL-2 was observed. Cryopreservation did not affect the composition or functionality of T-cell lines. *Summary/Conclusions.* Based on these results we propose a GMP-prove method for generation of pp65-specific T-cell lines using 6,6 μg/mL of pp65 protein for stimulation followed by isolation of specific T-cells based on IFN_ production. Isolated T-cells will be cultured for a short period on low dose IL-2 in order to retain maximal *in vivo* potential. This procedure yields GMP-grade T-cell lines comprising both CD8+ and CD4+ CMV-specific T-cells, which will be assessed for their clinical efficacy.

0446

FUNCTIONAL CHARACTERIZATION OF CYTOMEGALOVIRUS (CMV)-SPECIFIC CD4 AND CD8 T CELL LINES GENERATED BY USING PROTEIN-SPANNING POOLS OF PP65 AND IE1 DERIVED PEPTIDES

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Background and Aims. Reactivation of latent CMV in immunocompromised recipients of allogeneic stem cell transplantation remains a major cause of morbidity and mortality. Reconstitution of immunity by CMV-specific immunotherapy is an attractive alternative to standard treatment. In this study, we have analyzed the functional properties of CMV-specific T cells generated by using protein-spanning pools of CMV-derived peptides. *Methods.* Dendritic Cells (DC) were differentiated from donor peripheral blood monocytes after a 7-day culture in the presence of GM-CSF plus IL-4 and matured with TNFα, IFNα, IFNγ, IL1β, POLI I:C. Matured DC were then pulsed with a pool of 48 peptides spanning pp65, IE1, pp150 and pp50 proteins, which are recognised by both CD4 and CD8 T lymphocytes. Donor T cells were stimulated weekly three times at a T cell/DC ratio of 6:1 with mature peptide pulsed-DC. At the end of the culture T cells were analyzed for their phenotypic and functional features. *Results.* CD4 and CD8 CMV-specific T cell lines were successfully expanded from 10 CMV seropositive donors. Cultured T cells expressed CD8 (mean 70%, range 60-81%) and CD4 (mean 20%, range 15-28%) and showed a Effector Memory (mean 26%, range 19-30%) or a Effector Memory RA-Positive phenothype (mean 67%, range 59-77%). An enriched CMV-specific T cell population was observed after pentamers staining (7-45% pentamer-positive T cells). In all cases, cultured T cells showed a cytolitic activity against CD8-peptide pulsed target cells (average lysis 50%, range 40-55%) and, to a lesser extent, against CD4-peptide pulsed target cells (average lysis 35%, range 30-40%). In addition, cultured T lymphocytes were able to proliferate and to produce intracytoplasmic IFN-γ (average production 50%, range 35-60%) after exposure to peptide-pulsed DC. CMV-specific T cells were also analysed for the expression of adhesion molecules and chemokine receptors and for their ability to migrate in response to inflammatory (CXCL9, CCL3 and CCL5) and constitutive (CXCL12) chemokines. T cells showed high levels of CXCR3 (average expression 94%, range 81-99%), CCR1 (average expression 61%, range 57-92%), and to a lesser extent, CXCR4 (mean 25%, range 10-61%). In accordance with this profile, cultured T cells strongly migrated in response to CXCL12 (mean Migration Index (MI) 1.8, range 1.5-2), CCL5 (mean MI 7.2, range 5.4-11), CCL3 (mean MI 2.3, range 1.3-4.2) and CXCL9 (mean MI 2, range 1.5-2.7), which are involved in the recruitment of effector cells to peripheral sites of viral infection. Finally, CMV-specific T cells showed high level of CD49d (≥ 98%), which guides the extravasation of effector cells into inflamed tissues, and low levels of CD62L, a molecule involved in the migration to lymphoid organs. *Conclusions.* In conclusion, we demonstrated the possibility to generate activated and armed CMV-specific T cells, potentially able to reach viral-infected tissues and to recognize and kill CMV-infected cells.

0447

MICROBEADS AS ARTIFICIAL APCS AND CELL BRIDGES FOR T CELL CANCER THERAPY

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Cancer results from genetic alterations within tumor cells that promote their proliferation and resistance to apoptosis, but also to the failure of the immune system in the destruction of the tumor cells. Adoptive T cell therapy is achieving its first irrefutable successes in the reprogramming of immune responses against tumor cells. These successes are based on the grafting of anti-tumor T lymphocytes to hosts previously lymphodepleted by chemotherapy. We selected the B-CLL as a model to experiment this therapeutic strategy, because is the most common leukemia and is usually treated with lymphodepletive therapy. Furthermore, we and other have demonstrated that in these patients there is a reactive expansion of antileukemic T cells which maintain certain control over the growth of the leukemic cells. We have developed flow cytometry methods for the study of *in vitro* interactions between T cells and leukemic cells. These methods allow the accurate measurement of the growth and apoptosis of leukemic cells in coculture with T lymphocytes. Our laboratory is one of the pioneers in the use of microbeads coated with monoclonal antibodies (MAbs) in the polyclonal stimulation of T lymphocytes *in vitro*. Microbeads coated with Abs combinations can simultaneously stimulate the T cell antigen receptor and costimulatory receptors such as CD28 working like artificial antigen presenting cells that can be used to activate and expand antileukemic T cell clones. We are developing *in vitro* methodologies to break the immune tolerance to the leukemic B-CLL cells and to induce the cytotoxic T cell response against these tumor cells. The first strategy is to polyclonally stimulate (with anti-CD3, anti-CD28 and IL-2) T lymphocytes from these patients. A second and very promising strategy was pioneered by our laboratory. It uses microbeads to bridge leukemic cells and T cells. It uses one antibody against a leukemic cell antigen and other against costimulatory antigens of cytotoxic lymphocytes (anti-CD28). These microbeads link the leukemic cell and the cytotoxic T lymphocyte providing costimulatory signals for the T cell in the case it recognizes one antigen presented by the leukemic B cell. The application of methodologies of cell enumeration in coculture has demonstrated the efficacy of anti-CD23 and anti-CD28 coated microbeads in the stimulation of cytotoxic T cells to kill autologous leukemic B-CLL cells. We are now developing methods of generation of antileukemic effector T cells *in vitro*. We have demonstrated the efficacy of these cytotoxic T cells to kill autologous leukemic B cells *in vitro*. We also want to identify and select the T cell subsets with the highest potential for growth and the strongest killing activity against leukemic cells.

0448

THE IMMUNOMODULATORY EFFECTS OF HUMAN UNRESTRICTED SOMATIC STEM CELLS ON CORD BLOOD T CELLS

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Background. unrestricted somatic stem cells (USSCs) generation was initiated from fresh and cryopreserved cord blood. Reports are indicating that USSCs have unique immunologic properties, making them ideal for cellular therapy. USSCs are not immunogenic, they do not stimulate alloreactivity, and they escape lysis by cytotoxic T-cells and natural killer (NK)-cells. Thus, USSCs may be transplantable between HLA-mismatched individuals without the need for host immunosuppression. *Aims.* To evaluate probable immunomodulatory effects of USSCs on T cells proliferation. *Methods.* USSCs were plated in 96-well plates (2,000/well), and co-cultured for 3 days with T cells isolated from cord blood. In control group, cord blood T cells did not co-culture with USSCs. After cord blood T cells stimulated by PHA for 60 hours, T cell proliferation was assessed by MTT assay. Secretion of IFN-γ from stimulated cells was measured by ELISA kit. Expression of immunoregulatory molecules on USSCs was analyzed by flow cytometry. *Results.* USSCs expressed major histocompatibility complex (MHC) class I, lymphocyte function-associated antigen (LFA-3) constitutively and intercellular adhesion molecule (ICAM-1) antigens upon γ-interferon treatment but do not express CD80, CD86, or CD40 costimulatory molecules. The results from IFN-γ measurement showed that cord blood T cell proliferation was suppressed when 2,000 USSC were plated on each well. *Summary/Conclusions.* USSCs actively inhibit T-cell proliferation, suggesting that allogeneic USSCs transplantation might be accomplished without the need for significant host immunosuppression. USSCs transplantation may be use for modulation of immune system in hyper reactive and autoimmune diseases.

Myelodysplastic syndromes I

0449

VALIDATION OF THE NIJMEGEN PREDICTIVE SCORE FOR INDUCTION THERAPY IN MDS WITH HIGH-RISK MDS OR AML

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Background. Intensive Chemotherapy in high-risk MDS patients still is a matter of debate. The majority of MDS patients is too old to undergo induction chemotherapy, the relapse rate is high and the proportion of long-term survivors is with about 10-25% relatively low. Taking into account comorbidities, side effects and complications of the therapy as well as about 10% early death rates, one should try to select patients very carefully for intensive chemotherapy. Although it became clear that the initial karyotype predicts CR rate as well as long-term out-come (Knipp et al Blood, abstract 2004), it is not possible to use this as the only parameter to decide whether or not the patient should undergo induction therapy. The Nijmegen group re-cently proposed a predictive score that was calculated on the basis of a large amount of pa-tients (Osterveld, Leukemia Research, abstract 2005). Besides the karyotype, WBC, age, an-tecedent haematological malignancy and number of cytopenias were rated differentially to form 3 risk groups, associated with different long-term outcome. *Aims.* In order to validate this predictive score with an independent cohort of patients, who underwent induction therapy, we examined, whether the score was able to define risk groups. *Methods.* There were 283 patients with either high-risk MDS or MDS/AML or AML who were treated with induction at our institution between 1988 and 2005. Median age was 57 years (16-74). The patients received Induction therapy with Ara-C and an Antracyclin and patients younger than 60 years additionally received Etoposide. 16 patients underwent allografting after achievement of CR. 58% of the patients entered CR, 9% of the patients achieved PR, 23% of the patients had no remission and 10% of the patients died within 8 weeks after induction. *Results.* We then retrospectively tested the Nijmegen Score using this database. 25 patients were allocated to the low risk group (9%), 129 patients to the intermediate risk group (45%) and 129 patients to the high-risk group (46%). There was a difference in early death rate (0% vs. 6% vs. 15%, p=0.002). The overall survival was 40 months in the low, 24 month in the intermediate and 12 months in the high-risk group (p<0.00005). The difference between intermediate and high-risk group was also statistically significant (p<0.00005). The percentage of patients still alive two years after induction was 75% vs. 48% vs. 27%, and after 5 years 50% vs. 23% vs. 7%. Only within the low-risk group, there are patients with a long-term survival up to 16 years. *Conclusions.* These data indicate that a) the Nijmegen Score in validated in a large independent patient group and b) patients aged above 55-60 years in the high-risk group should not undergo induction therapy but should be treated either with epigenetic drugs, farnesyltransferase inhibi-tors, or when presenting with 5q- with Lenalidomide.

0450

LENALIDOMIDE IN DEL(5Q) MDS PATIENTS: DFFERENT PATTERNS OF RESPONSE

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The novel amino-substituted thalidomide analogue lenalidomide (Revlimid®) has recently been approved in the USA for the treatment of transfusion dependent anemia due to low- or intermediate-1-risk myelodysplastic syndromes associated with a del(5q) cytogenetic abnormality with or without additional chromosomal abnormalities. The decision was based on data of a phase II study on a total of 148 del(5q) patients that showed an impressive number of cytogenetic remissions and transfusion independence. Given that 15% of patients with MDS bear the del(5q) abnormality, and that 12.000 to 15.000 new MDS cases are diagnosed yearly in the European Union, up to 2.300 new patients every year will be eligible for treatment with lenalidomide. In this presentation, we give an overview of treatment approaches based on our experience gained on more than 40 patients treated with lenalidomide at a single center. An important number of patients have different patterns of response to the drug the attending physician must be familiar with to avoid under- or overtreatment and to assure the best possible care. Five response types are described: First, the uncomplicated responder who does not need drug dose reduction and goes into long-term hematological and cytogenetic remission. Second, the typical responder who does require dose reduction but still achieves long-term remission. Third, the intermittent responder needs careful long-term blood count monitoring and individual dosing. This type of responder is characterized by an initial beautiful response with both transfusion independence and cytogenetic response that suddenly vanishes and leads to recurrence of initial transfusion dependence. Some of these patients benefit from a simple drug holiday, some from resuming lenalidomide after a varying drug interruption. Fourth, there are very few late responders who achieve transfusion independence weeks after stopping the drug for lack of efficiency. Finally, some patients simply do not respond to the drug without us being able to identify predictive factors which patients belong to this group. It is interesting to note that cytogenetic remission is not a prerequisite for long-term response. Some patients remain del(5q) positive with a long-term ongoing hematologic remission. On the other hand, we will also report our experience in patients with complex cytogenetic aberrations who seem to do as well as 5q-syndrome patients. Regarding adverse events, we will present our experience in treating skin reactions, pruritus and scalp itching, muscle cramps, diarrhea, hypothyroidism, and of course neutropenia and thrombocytopenia. None of our patients required platelet transfusions because we regularly interrupted treatment at values of 50.000/µl. Grade 4 neutropenia is common, but only the minority of patients needed granulocyte stimulating cytokines. Titrating the drug until neutropenia and thrombocytopenia occur has proven effective in achieving erythroid response during regeneration of haematopoiesis. We conclude that lenalidomide is a reasonably safe drug in the del(5q) patient population that is characterized by a number of specific response patterns. Knowing these characteristics will enable the physician to safely induce remissions in an important part of patients.

0451

HIGH INCIDENCE OF THERAPY-RELATED MYELODYSPLASIA AND ACUTE LEUKAEMIA IN PATIENTS TREATED WITH FLUDARABINE AND CYCLOPHOSPHAMIDE FOR INDOLENT LYMPHOPROLIFERATIVE DISORDERS

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Therapy-related myelodysplasia and acute leukaemia (tMDS/tAML) are late complications of chemotherapy, with increased risk associated with advancing age. tMDS/tAML has recently been reported following fludarabine in large trials (median age about 60). We analysed the incidence of tMDS/tAML in all patients in our unselected general haematology clinic (median age 72) who had received fludarabine for indolent lymphoproliferative disorders over the last 11 years. The blood counts of all patients at analysis or death were investigated. Bone marrows were then examined on all patients with persistent cytopenias (defined as Hb<8gm/dL, neutrophils<1.0×10^9/L, platelets<50×10^9/L persisting more than 10 weeks after chemotherapy). Marrows were examined independently by 3 Haematologists. All cytotoxic drugs administered and total dose of fludarabine were recorded for all patients. Of 57 patients, 41 survived more than 12 months following fludarabine. 8/41 (20%) patients (median age 73) developed tMDS/tAML at a median of 22 months (range 12-43) from first fludarabine treatment (actuarial incidence 33% (95%CI:22-42%)). 6/8 died from their tMDS/tAML, 1 died of complications post stem cell transplant and 1 died of carcinoma of pancreas. All 8 died with the underlying lymphoproliferative disease under control. 4 cases were recognised prospectively, and 4 further cases were recognised after death. All patients developing tMDS/tAML had received fludarabine (F) together with cyclophosphamide (C). The patients developing tMDS/tAML had received a significantly higher dose of fludarabine than those not developing tMDS/tAML (p=0.01, see Table). The difference in fludarabine dose was greater comparing the tMDS/tAML patients with the subgroup of patients who also received fludarabine and cyclophosphamide and did not develop tMDS/tAML (p=0.005). (When fludarabine was given with cyclophosphamide, most patients received 10 mg cyclophosphamide to every 1mg fludarabine given). The incidence of tMDS/tAML increases markedly with age. The older age of our patients is likely to be a cause for our high incidence. There was a relationship between the dose of fludarabine administered and the development of tMDS/tAML. Reports of tMDS/tAML following fludarabine exposure demonstrate cytogenetic changes similar to

those induced by alkylating agents. Since combining 2 alkylating agents causes a very high risk of tMDS/tAML, the combination of fludarabine and cyclophosphamide may be highly leukaemogenic. Our data support this hypothesis. The recognition of 50% of our cases retrospectively, is in line with emerging trial data which suggest that tMDS/tAML may be missed unless specifically looked for. In summary we have shown a high incidence of tMDS/tAML in elderly patients treated with fludarabine and cyclophosphamide, with a clear relationship to fludarabine dose. The true extent of tMDS/tAML may be under-recognised. The best approach in the predominantly palliative treatment of the elderly may be to exercise caution with the combination of fludarabine and cyclophosphamide, and to reduce total cytotoxic drug exposure.

Table 1. Comparison between patient groups who did and did not develop tMDS/tAML.

Patient group	*Median age in years (range)*	*Median dose F mg/m²(range)*	*p value for difference between F dose (tMDS/tAML vs non-tMDS/tAML)*	*Median no of other chemotherapy given (range)*
tMDS/tAML patients (8) (alla had F and C)	73 (62-82)	541 (350-703)		2 (1-3)
Non-tMDS/tAML patients (33)	72 (42-83)	300 (64-1038)	p=0.01	2 (0-4)
Subgroup non-tMDS/tAML patients who had F alone (25)	69 (46-83)	270 (64-1038)	p=0.005	
Subgroup non-tMDS/tAML patients who had F alone (7)	72 (42-79)	375 (100-690)		

1 patient had fludarabine, mitoxantrone and dexamethasone.

0452

IMPAIRED SDF-1-INDUCED MIGRATION OF CD34+ CELLS FROM MDS PATIENTS AS A RESULT OF DECREASED ACTIVATION OF PROTEIN KINASE B, RAC AND F-ACTIN POLYMERISATION

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Background. SDF-1 is a chemotactic agent that is implicated in mobilisation, retention and homing of haematopoietic stem cells. It was recently described that CD34+ cells from patients with myelodysplasia (MDS) demonstrate an impaired SDF-1-mediated migration, despite the presence of high levels of SDF-1 in the bone marrow. *Aims.* To investigate the molecular mechanisms underlying the decreased SDF1-induced migration of CD34+ cells from low risk MDS patients. *Methods.* Migratory behaviour of sorted CD34+ cells was assessed using Transwell assays. Phosphorylation status of protein kinase B (PKB) and extracellular signal-regulated kinase (ERK1/2) were analyzed by Western blot analysis. Rac activation was studied by performing pull down assays. *Results.* We confirmed the reduced migratory capacity of MDS progenitors towards SDF1 compared to normal CD34+ cells (5.7%±2 vs 10%±2, n=7, p=0.02). This defect could not be attributed to lower SDF-1-receptor expression, as the number of CXCR4-expressing cells was similar between MDS and normal CD34+ cells (16%± 3 vs 16%±1, n=6, p=0.5). It has been described that the rate of F-actin polymerisation might be limiting for cell migration. We therefore examined F-actin polymerisation in response to SDF-1 and showed that this was significantly reduced in MDS CD34+ cells compared to normal CD34+ cells (151±5 vs 176±8, n=6, p=0.03). To further elucidate the molecular mechanisms of the impaired migration we investigated the involvement of the phosphatidylinositol 3 kinase (PI3K), ERK1/2 and Rac signalling pathways in migration and actin polymerisation of normal CD34+ cells. Pre-treatment of CD34+ cells with the ERK inhibitor U0126 lead to significant decrease in migration (21%), as did inhibition of PI3K pathway with LY294002 (50%). Incubation of CD34+ cells with the Rac inhibitor NSC23766 abrogated SDF-1-induced migration consistently (61%). However, U0126 and LY294002 treatment did not affect the SDF-1-induced F-actin polymerisation of CD34+ cells, whereas incubation of CD34+ cells with NSC23766 did attenuate actin assembly in response to SDF-1 (191±16 vs 137±6, p=0.04). We subsequently questioned whether the disturbed migration of MDS CD34+ cells is in part the result of an ineffective activation of one of these signalling pathways. Investigation of phosphorylation status of ERK1/2 and the PI3K target PKB in response to SDF1 showed that ERK1/2 activation was decreased in 3 out of 6 patients, whereas PKB activation was impaired in 5 out of 6 patients when compared to normal CD34+ cells. Furthermore, levels of Rac-GTP, corrected for total levels of Rac present in the lysates, were lower in SDF-1 stimulated progenitors from MDS patients compared to healthy controls (n=3). *Conclusion.* These results indicate that although ERK1/2, PI3K and Rac are involved in CD34+ migration towards a SDF-1 chemotactic gradient, only Rac is necessary for F-actin polymerisation in response to SDF-1. It is conceivable that at least two separate pathways are required for migration; a PI3K-dependent pathway and a PI3K-independent Rac-actin pathway. Both of these pathways appear to be impaired in MDS CD34+ cells, resulting in a decreased SDF-1-induced migration.

0453

EVI-1 GENE EXPRESSION IN MYELODYSPLASTIC SYNDROMES: QUANTITATIVE ASSESSEMENT AND *IN VITRO* MODULATION INDUCED BY ARSENIC TRIOXIDE

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Background. The EVI-1 gene is located in chromosome 3q26 and codes for a zinc finger protein which acts as transcriptional repressor. Rearrangements of the EVI-1 locus in chromosome band 3q26 are associated with poor prognosis in myeloid malignancies. The overexpression of the gene has been described in a subset of patients without evidence of gene rearrangements. Moreover it was suggested that the overexpression of EVI-1 gene confers a high degree of sensitivity to Arsenic trioxide therapy. *Aims.* The aim of the study was to analyze the expression level of EVI1 in a large number of different subtypes of myelodisplastic syndromes and to study the *in vitro* effects of arsenic trioxide. *Methods.* We analyzed the expression levels of EVI-1 in 230 BM samples and 162 PB collected from 345 MDS patients. 126 were refractory anaemias (RA), 144 refractory anaemias with excess of blasts (RAEB) and 75 RAEB-T or secondary AML (s-AML). Moreover we tested 37 *de novo* AML patients and 22 BM and 35 PB samples obtained from healthy volunteers. 4 AML and 1 MDS patients showed the 3q26 rearrangement detected by cytogenetic analysis. The expression level of EVI-1 was established using quantitative Real-Time PCR based on a specific primer and probe set (Assays-on-Demand, Applied Byosystems). The values obtained were normalized using ABL as housekeeping gene and the final results were expressed using the DeltaDelta Ct method. 80 BM MNCs were incubated with 1 micromolar arsenic trioxide and proliferation and colony growth were evaluated. *Results.* We detected very low levels of EVI-1 expression in BM (mean value of 2-DeltaDeltaCt = range 0-11) and undetectable levels in PB. By contrast, in 134 patients out of 345 (39%) abnormal levels of EVI-1 were detected. Significantly higher levels were found in patients with 3q26 rearrangements (range 294-35120). The patients expressing high levels of EVI-1 were distributed as follow: 38 RA (mean value of Delta Delta Ct 49; range 11- 64), 62 RAEB (mean value= 123; range 60-264) and 34 RAEB-T (mean= 2196; range 162-6653). 5 out of 37 *de novo* AML showed abnormal expression of EVI-1 (mean value 468 range 56- 530). EVI-1 expression was evaluated during follow-up of twelve patients who converted into overt leukaemia and in all the cases EVI-1 levels increased during progression. *in vitro* treatment with arsenic trioxide induces in 22 out of 80 samples (28%) a significant increase of BFU-E colony number, and this was observed mainly in patients characterized by high EVI-1 levels (17 out of 22). Moreover a significant reduction of EVI-1 gene expression was observed after arsenic trioxide incubation (p=0,003) as compared to controls. *Conclusions.* These data allow to establish that the overexpression of EVI-1 is present in 39% of MDS patients regardless of the presence of the 3q26 rearrangement. The overexpression seems to be more frequent in RAEB and s-AML respect to RA and it increases during disease progression. The arsenic trioxide treatment induces reduction of EVI-1 transcript amount and a significant increase of BFU-E growth in patients overexpressing EVI-1.

0454

CYTOGENETIC EVOLUTION IN DEL(5Q) MYELODYSPLASTIC SYNDROME

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Myelodysplastic syndromes are clonal hematopoietic stem cell disorders that are characterized by a wide prognostic heterogeneity. While some MDS like the pure sideroblastic anemias tend to remain stable for years, others evolve to higher risk MDS or acute myeloid leukaemia. During disease progression, cytogenetic evolution occurs and is an independent prognostic risk factor. To assess cytogenetic evolution in MDS with del(5q), we analysed data of 33 patients with *de novo* del(5q) MDS. Inclusion criteria were del(5q31.1) MDS at initial cytogenetic investigation in at least two metaphases irrespective of International Prognostic Scoring System (IPSS) rating. Cytogenetic evolution was defined as appearance of additional chromosomal abnormalities within the initial del(5q) clone. Gain of chromosomes was accepted if recorded in at least two metaphases, loss of chromosomal material had to be evident in at least three metaphases according to ISCN rules, or if single cell abnormalities were confirmed by FISH analyses. Additional clones with chromosome aberrations other than del(5q) were regarded as unrelated clones but not as cytogenetic evolution within the del(5q) clone. Initial cytogenetics were performed between 1989 and 2003. The last follow-up investigation was done in 2004 by the reference cytogenetic center of the German MDS study group (BS). Median age of the 33 patients was 62.2 years (range, 32 to 83). All patients had at least two cytogenetic evaluations (range, 2 to 9) with a median time between first and last examinations of 36 months (range, 3 to 172). 19 patients had RA according to FAB, 4 had RARS, 8 RAEB, and 2 were not classifiable. 2 patients (6%) acquired additional cytogenetic lesions within the initial del(5q) clone. These were t(1;3)(p33;p14) after 25 months, and inv(3)(q13q25) after 43 months. One patient had a trisomy 8 in a different clone in a previous examination in 8 metaphases that was inapparent at follow-up in 20 metaphases after 32 months. One patient with RAEB who had initially additional trisomy 21 in the del(5q) clone acquired a second clone with a der(18;21). All true cytogenetic evolutions occurred in 5q-syndrome patients. One of the two patients with cytogenetic evolution did not respond to lenalidomide treatment. However, karyotype complexity in del(5q) MDS does not seem to impact on response to lenalidomide therapy as there is increasing evidence that del(5q) patients with complex karyotype have the same amount of cytogenetic remission rates as 5q-syndrome patients. As a conclusion, del(5q) MDS display long-term clonal stability, with cytogenetic evolution being evidenced in less than 10% of patients. Clonal evolution is not necessarily linked to advanced MDS at initial presentation.

0455

OVEREXPRESSION OF MATRIX METALLOPROTEINASES 2 AND 9 IN MYELODYSPLASTIC SYNDROMES

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Matrix metalloproteinases (MMP) are a family of zinc-dependent endopeptidases which are able to degrade all the protein components of the extracellular matrix. MMP-2 (type IV collagenase, gelatinase A) and MMP-9 (type V collagenase, gelatinase B) have been implicated in tumor progression and metastasis and, recently, it was suggested that these enzymes may also contribute to leukemic dissemination. We analyzed the expression of MMP-2 and MMP-9 in bone marrow cells from 129 patients with myelodysplastic syndrome (MDS) (49 RA, 26 RARS, 32 RAEB, 3 RAEB-t, 19 CMML), not previously treated, and from 45 non hemopathic subjects, in order to evaluate whether abnormalities in their expression were associated with relevant laboratory and clinical findings. Moreover, a possible correlation was investigated between MMP positivity and altered apoptosis level, as measured by TUNEL technique, or altered proliferative activity, as evaluated by MIB-1 immunostaining. MMP-2 and MMP-9 were detected on bone marrow smears by an immunoalkaline phosphatase method (streptavidin-biotin complex) using primary murine monoclonal antibodies raised against human MMP-2 (clone A-Gel VC2, NeoMarkers) and human MMP-9 (clone IIA5, NeoMarkers). In normal samples MMP-2 was detected only in occasional myeloid cells, whereas MMP-9 was expressed in some 20-30% of maturing myeloid cells. In MDS the percentages of cells positive for MMP-2 (median 36%, IQR 22-47%) and MMP-9 (median 40%, IQR 28-53%) were significantly higher than those observed in normal controls (p=0.0000 and 0.04 respectively). There was a close relationship between MMP-2 and MMP-9 expression in MDS myeloid cells (p=0.003) and also many erythroblasts expressed both enzymes. In early MDS (RA and RARS) percentages of MMP-2 and MMP-9 positive cells higher than in advanced forms were observed (p=0.01 and 0.001 respectively). In advanced MDS a tendential inverse correlation between MMP-2 and TUNEL positivity was identified by the Spearman correlation test (p=0.06), whereas in the whole MDS group MMP-9 as well as MMP-2 expression was independent of the proliferative rate. A significant inverse correlation between either MMP-2 or MMP-9 and bone marrow blast cell percentage was observed (p=0.03 and 0.01 respectively), but no significant relationship was found between MMP levels and clinical and laboratory features such as age, leukocyte count or karyotype. A low MMP expression was associated with significantly shorter overall survival. Seven patients with early MDS were treated with thalidomide. Very interestingly, thalidomide treatment decreased the cellular expression of MMP-2 and MMP-9 as well as apoptosis in bone marrow erythroblasts of responsive cases. In conclusion, for the first time the relation between MMP abnormal expression profile and other biological and clinical features has been evaluated systematically in MDS. Our findings suggest that the production and release of these enzymes may influence hematopoietic cell death and behaviour, possibly by the processing of regulatory proteins in marrow, with a potential prognostic significance for disease progression. On the other hand, MMPs may represent specific targets for therapeutic intervention.

0456

A ROLE FOR THE ENDOPLASMIC RETICULUM AND THE MITOCHONDRION IN ERYTHROID CELL APOPTOSIS THAT CHARACTERISES LOW GRADE MYELODYSPLASTIC SYNDROMES

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Cell death by apoptosis was shown to account for the ineffective erythropoiesis that characterises low grade myelodysplastic syndromes (MDS). We have shown previously that the death receptor Fas was overexpressed at the surface of MDS erythroid precursors. Using an *ex vivo* liquid culture to analyse the differentiation of low grade MDS CD34+ cells into red cells, we demonstrated that apoptosis of MDS erythroid precursors could be prevented by either Fas-Fc or the ectopic expression of a dominant negative mutant of the adapter molecule FADD (Fas-associated death domain), a component of the death-inducing signalling complex that mediates death receptor-induced apoptosis. We and others also showed that the release of mitochondrial cytochrome c participated to this process, suggesting a connection of the extrinsic to the intrinsic pathway of apoptosis. To further address this latter question, we over-expressed the anti-apoptotic Bcl-2 protein in CD34+ bone-marrow cells before inducing their erythroid differentiation. For that purpose, we used lentiviral constructs including cDNA encoding either wild-type Flag-Bcl-2 (WT) or a Flag-Bcl-2 targeted to endoplasmic reticulum (ER) by a cytochrome b5 sequence. These constructs, which also encoded eGFP under the control of an IRES, were used to infect either control (n=10) or low grade MDS (n = 15) $CD34^+$ bone-marrow cells. These cells were subsequently allowed to grow along the erythroid lineage during 14 days. Phosphatidylserine exposure at the cell surface and mitochondrial membrane permeability (MMP) were increased in MDS compared to control erythroid precursors. The increase in MMP was associated with enhanced cytochrome c release and caspase-9 activation. Infection of CD34+ cells with the two Bcl-2-encoding lentiviral constructs induced the expression of Bcl-2 protein in more than 80% of control and MDS erythroid precursors, compared to less than 10% of those infected with the control vector. Specific targeting of Bcl-2 to the ER was confirmed by both immunofluorescence analyses and the lack of inhibition of lonidamide-induced cell death. Overexpression of Bcl-2 WT which is located in mitochondria and ER, or overexpression of Bcl-2 ER delayed the erythroid differentiation of both MDS and normal cells. In contrast, the two Bcl-2 encoding viruses specifically inhibited phosphatidylserine externalisation, MMP decrease and caspase-9 and -3 activation associated with erythroid differentiation of MDS bone-mar-

row CD34+ cells. Interestingly, over-expressed Bcl-2, either wild-type or ER-targeted, failed to affect the truncation of the BH3-only protein Bid. This suggests that the mitochondrial pathway of apoptosis is activated downstream of Fas through Bid. Over-expressed Bcl-2 decreased also the release of ER calcium in response to thapsigargin, reflecting lower intracellular calcium stores and a less apoptosis. Altogether, these results confirm the involvement of mitochondria in erythroid cell death that characterises low grade MDS and strongly argue for a participation of ER in this apoptotic pathway, both organelles acting downstream of the death receptors.

0457

ISSUES AFFECTING QUALITY-OF-LIFE IN PATIENTS LIVING WITH MYELODYSPLASTIC SYNDROMES: RESULTS OF PATIENT FORUM DISCUSSIONS IN EUROPE

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Background. Patients living with MDS experience significant deterioration in their quality-of-life. New treatments (approved and under development) for MDS have provided patients with hope that this deterioration in quality-of-life can be limited or eliminated in the near future. *Aims.* Patient forum discussions were conducted with the goals of determining the key issues regarding quality-of-life (QoL) in patients living with MDS including: feelings about the attitude of, and support offered, by health care providers, the patient's depth of knowledge about MDS, and effect of treatment on QoL. Educational programming will be developed based on the information derived from these forums. *Methods.* Nine MDS Foundation Centers of Excellence volunteered to participate and 4 forums convened to date. Questionnaires were developed, vetted, and translated by participating sites. Questionnaires were consistent in all locations. Discussion focus varied due to the free-flowing nature of the forums. These forums were conducted in Edinburgh, Paris, Bournemouth (England), and London. *Results.* A total of 67 patients and 92 caregivers participated. Patient sample was Caucasian (100%); male (52%); female (48%); Age range <50 (10%), 50-75 (57%), >75 (33%). 5 have less than 6 years of education, 30 had 10-12 years, and 32 had >12 years. 50 are married (75%) and the 95% live with other people. 21% are employed full or part time and 50% are retired with 29% unknown. Patient QoL experiences were similar between sites and reflected substantial feelings of life disruption due to MDS and time required for disease management. Physician visits, testing, transfusions, treatment, travel time, and symptom/adverse event management contributed to feelings of *lose of life control*. Fatigue is the issue affecting QoL most often - impacting patient's ability to perform activities of daily living, work, and participation in social and family life. Emotional well being is significantly decreased and described as *waiting for something to happen*. Physician relationships at the COEs were viewed positively by the majority while relationships with community physicians were viewed in a negative context due to physician's lack of knowledge. Patients described time spent *educating the doctor*. Nurses were viewed as key to patient's knowledge and well being. Patients expressed overall satisfaction with current treatment however 65% felt that new drugs were not being made available quickly enough within the EU. Transfusions, in tandem with chelation therapy, were viewed as impacting QoL significantly second only to fatigue. Patients viewed transfusions as a necessary evil to deal with their fatigue. Caregivers expressed a need for information to assist them in dealing with family/friends with MDS. *Conclusion.* MDS has a substantial impact on patients QoL including interactions with family and friends. Physicians and other healthcare professionals should be aware of this impact and attempt to provide patients with information and options to lessen the burden of this disease and minimize its impact. New treatment options should be explored with patients, including participation in clinical trials, with the goal of improving QoL and lessening the disease burden by potential reduction or elimination of transfusions, lessening fatigue, oral medications for chelation therapy.

0458

FOUR DIFFERENT TYPES OF MDS PATIENTS WITH 5Q- ANOMALIES

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Background. About 50% of all MDS patients show karyotype aberrations at the time of di-agnosis. The most frequent chromosomal anomaly is the del (5q) aberration. The WHO rec-ognized the 5q- Syndrome as a separate entity within the group of myelodysplastic disor-ders. However, a high number of MDS patients show 5q- aberrations together with another abnormality or within a complex karyotype. *Aims.* A better description of the different types of 5q- aberrations in warranted. *Methods.* We screened the German MDS registry Düsseldorf for patients presenting with 5q- aberrations, regardless of WHO type and other chromosomal aberrations. *Results.* Out of 2897 patients in the registry, 1038 patients have been karyotyped at diagno-sis (36%). 180 of them (17%) showed a 5q- anomaly either alone or in combination with other aberrations. We then separated the patients into 4 groups: 5q- Syndrome (del(5q) as a single anomaly, medullary blast count <4, Group A)), del(5q) as a single anomaly with elevated blasts (Group B), del(5q) with an additional chromosomal abnormality (group C) and del(5q)-MDS within a complex karyotype (group D). We then examined haematological data and prognosis of the 4 groups. For calculating the prognosis, patients who underwent Induction therapy, allogeneic stem cell transplantation or Revlimid treatment were censored. Per definition, all patients in groups A had a medullary blast count of <5%, and all patients in group B had RAEB or RAEB-T. Group C consisted of RA and RARS in 83%. 68% of the patients in group D had RAEB or RAEB-T. The degree of hematopoietic insufficiency was more pronounced from in B, C and D and the prognosis was adverse in group B, C and D. Median survival of group A was 85 months as compared to 63, 31 and 7 months respectively ($p<0.005$). The cumulative risk of AML was 13% in the group A, as compared to 33%, 42% and 55% ($p<0.05$). *Conclusions.* These data show that the prognostic impact of 5q- anomaly as well as its pathophysiologic impact is heavily influenced by other factors, such as medullary blast count and additional aberrations. This should be taken into account, when assessing the prognosis is planning treatment for those patients.

0459

THE PROGNOSTIC MEANING OF INVOLVEMENT OF 5Q- ABERRATIONS IN PATIENTS WITH MDS AND COMPLEX KARYOYTPE

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Background. It is well known, that patients with Myelodysplastic syndromes with a complex karyotype have a very poor prognosis, facing a median survival of less than 1 year. There is ample evidence on the efficacy of lenalidomide in patients with del(5q), not only in 5q- Ssyndrome but also in patients with del(5q) as part of a complex karyotype. *Aims.* In order to examine the prognostic role of del(5q) involvement within complex karyo-types, we screened the German MDS registry Düsseldorf. *Methods.* A complex karyotype was defined according to the IPSS definitions. 155 patients were diagnosed with a complex karyotype. Median age was 66 years (18-89). There were 3 patients with RA, 25 with RCMD, 18 with RCMSDRS, 27 with RAEB I, 38 with RAEB II, 7 with CMML and 37 with RAEB-T. 45 patients have been treated either with induction chemotherapy or underwent allogeneic stem cell transplantation. These patients were ex-cluded from calculations of prognosis. *Results.* We then separated the patients into a group with involvement of del(5q) (n=53) and a group, in which del(5q) was not part of the complex karyotype (n=102). The distribution of both groups to WHO types did not differ significantly. Clinical and hematological charac-teristics (haemoglobin, platelet count, WBC, ANC, LDH) were not different between the groups; median age at diagnosis was 63 years in the non-del(5q) group and 66 years in the del(5q) group. The median survival of the del(5q) group was 7 months as compared to 14 months in the patients without 5q-involvement ($p=0.006$). 12 months after diagnosis, 22% of the del(5q) patients were alive, as compared to 49% of the non-del(5q) patients. 77% of the del(5q) pa-tients and 73% of the non del(5q)

patients died disease-related (AML, infections, bleeding). The risk of AML evolution 1 and 2 years after diagnosis was 60 and 74% in the del(5q)- group and 44 and 50% in the non del(5q) group (p=n.s.). The overall percentage of patients that developed AML was not different (53% vs 55%, p=n.s.). *Conclusions.* Del(5q) is associated with an extreme poor prognosis when diagnosed within a complex karyotype. Because only a minority of these patients can undergo a curative ap-proach, the efficacy of lenalidomide should be studied in this patients group the near future.

0460

MRI T2* MEASUREMENTS SHOW NO MYOCARDIAL IRON LOADING IN PATIENTS WITH MYELODYSPLASIA ON LONG-TERM BLOOD TRANSFUSION THERAPY

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Background. Iron overload is inevitable in chronic transfusion-dependent anemia. Excess iron can accumulate in the liver, endocrine organs and heart which may result in end-organ damage. Mortality from iron overload in thalassaemia is dependent on the magnitude of myocardial iron load. It is standard practice to initiate iron chelation therapy early in children with chronic transfusion-dependent anemia because it improves their life expectancy. Myelodysplastic syndromes (MDS) also result in chronic transfusion-dependent anemia but affects older adults. However, there is no data showing improved life expectancy with iron chelation therapy in this group of older patients with significant co-morbidities. Accurate measurement of iron overload requires tissue biopsy, which carries a high risk of major bleeding in patients with myelodysplastic syndromes. Serum ferritin is an indirect surrogate marker for iron overload but lacks precision and reliability. MRI T2* measures iron load in liver and myocardium. The accuracy and reliability of this method has been validated for hepatic and myocardial iron load. *Aims.* 1. Assess hepatic and myocardial iron load using MRI T2* in chronic transfusion-dependent good-prognostic MDS patients. 2. Correlate hepatic and myocardial iron loading to the duration of transfusion therapy, serum ferritin levels, and iron chelation therapy. 3. Correlate cardiac function to iron load. *Methods.* Good prognostic MDS patients on long-term transfusion therapy were identified using IPSS scoring system. Liver function tests and serum ferritin were monitored at regular intervals in all patients. Magnetic resonance imaging (MRI) of heart and liver was performed to assess hepatic and myocardial iron loading and cardiac function, in patients with serum ferritin greater than 1000 mcg/L. Hepatic and myocardial iron load was classified as none, mild, moderate and severe based on MRI T2* values (see Table 1).

Table 1.

Hepatic T2 (ms)*	*Hepatic iron overload*	*Myocardial T2* (ms)*	*Myocardial iron overload*
≥ 6.3	None	≥20	None
6.3-2.7	Mild	20-12	Mild
2.7-1.4	Moderate	12-8	Moderate
≤ 1.4	Severe	≤ 8	Severe

Cardiac function assessment included measurement of left ventricular ejection fraction (LVEF) using MRI. We report the results obtained in 7 patients who have completed the study. The median number of red cell units transfused was 106 units (range 42-257 units). The median duration of blood transfusions was 3 years (range 2-12 years). The median serum ferritin level at the time of study was 4400 mcg/L (1560-6651 mcg/L). Six patients were receiving iron chelation therapy at the time of study. None of the 7 patients had clinical features of hepatic insufficiency at the time of the study. *Results.* 1. Moderate hepatic iron overload was detected by MRI T2* measurements in all patients on chronic transfusion therapy. 2. Raised serum ferritin levels correlated with hepatic iron overload. 3. Myocardial iron overload was absent by MRI T2* measurements in all patients. 4. Cardiac LVEF was normal in all patients. *Conclusions.* 1. Myocardial iron overload was not detected by MRI T2* in multi-transfused good prognostic MDS patients. 2. Myocardial iron load did not correlate with hepatic iron load or serum ferritin level. 3. Larger studies are needed to clarify these findings and to determine the role of iron chelation therapy in these patients.

0461

EVIDENCE FOR A ROLE OF CD40 IN THE PATHOGENESIS OF LOW-RISK MYELODYSPLASTIC SYNDROMES

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Background and aim of the study. Myelodysplastic syndromes (MDS) form a heterogeneous group of clonal haematopoietic disorders characterized by peripheral cytopenia, marrow dysplasia and an increased risk to develop AML. There is increasing evidence that the cytopenias in early phase MDS are partially caused by immunological mechanisms. The aim of this study was to investigate if the CD40-CD40L interaction plays a role in the pathogenesis of MDS-related bone marrow failure. Our hypothesis is based upon the knowledge that the interaction between CD40 and its natural ligand CD40L (CD154) is involved in normal immune responses, but also in the pathogenesis of several non-hematological disorders. *Methods.* 1/ With FACS we measured the expression levels of CD40 on CD14+ monocytes and CD40L on CD4+/CD3+ lymphocytes in PB samples of 18 untreated and non-transfused MDS patients (10 RA, 8 RARS) and 12 controls. 2/ CD14+ cells were isolated from PB from 17 patients (14 RA, 2 RARS, 1 RAEB) and 19 controls with MACS columns and cultured for 7 days in IMDM + 15% fetal bovine serum. They were subsequently stimulated for 24h with lipopolysaccharide (LPS) 1.0 µg/ml or agonist monoclonal anti CD40 antibody (clone 64, Bioceros NV, Netherlands) at a concentration of 10 µg/mL. TNF-α concentrations in supernatants were measured with ELISA. 3/ Bone marrow MNC of 11 patients (5 RA, 2 RARS, 3 RAEB and 1 RAEB-t) were cultured in methylcellulose + growth-factors (M4434, Stem Cell Technologies) in the presence or absence of 10 µg/ml 5D12 (antagonist chimeric monoclonal anti-human CD40 antibody, Bioceros NV, The Netherlands). *Results.* 1/ MDS patients had a significantly higher percentage of circulating CD40+/CD14+ (9.40% ± 2.05 vs. 1.89% ± 0.55, p=0.0125), and CD40L+/CD4+/CD3+ cells (5.65 ± 2.76 vs. 3.99 ± 1.34, p=0.049) compared to controls. 2/ CD40-ligation, but not LPS, induced a significantly higher TNF-α production in patients compared to controls (388 ± 516 vs. 83 ± 24 pg/ml, p=0.0065). In patients, TNF-α production after CD40 stimulation was also significantly higher than after stimulation with LPS (388 ± 516 vs. 64 ± 139 pg/mL, p=0.016). In controls, TNF-α levels after LPS or CD40 stimulation were comparable. 3/ Co-culture of MDS bone marrow MNCs with 5D12 increased *in vitro* colony formation (141 ± 48 vs. 116 ± 43, p=0.067), an effect not observed in controls. *Conclusion.* We conclude from these observations that CD40-CD40L interactions might play a role in the pathogenesis of MDS-related bone marrow failure. This is supported by the observation of an increased number of circulating CD40+/CD14+ monocytes and CD40L+/CD4+/CD3+ lymphocytes in MDS patients. We have also shown that CD40-ligation induces a significantly higher TNF-α production by monocytes from patients compared to healthy volunteers. Finally, we have preliminary evidence that blocking the CD40-receptor can increase colony formation *in vitro*. These results mark a possible new target to treat cytopenias in MDS.

0462

IMMUNOPHENOTYPIC ANALYSIS OF CD34+ CELL SUBSETS IN BONE MARROW SAMPLES FROM MYELODYSPLASTIC SYNDROME PATIENTS

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Introduction. Current investigations regarding the presence of phenotypic aberrations among CD34+ cells in patients with myelodysplastic syndrome (MDS) have provided limited information about their frequency and subtypes due to the use of limited monoclonal antibody panels and/or a evaluation of insufficient amounts of CD34+ bone marrow (BM) cells. *Objective.* Our aim was to phenotypically characterize the myeloid and lymphoid CD34+ BM cell compartments in patients with MDS using a large panel of monoclonal antibodies in order to identify phenotypic alterations that may be useful for the diagnosis and classification of the disease. *Material and Methods.* Overall we studied 63 BM samples corresponding to 11 normal BM (NBM) and 52 BM from patients with MDS (including 19 low risk MDS patients (LR-MDS) and 33 high risk MDS cases (HR-MDS). CD34+ cells were identified and sub-

clasified into three different subsets based on their immunophenotypic features: 1) immature precursors (IP), neutrophyl precursors (NP) and lymphoid precursors (LP). Each CD34+ subset was characterized for its light scatter characteristics and the expression of multiple antigens as analyzed through the use of quadruple combinations of monoclonal antibodies measured by flow cytometry. *Results.* All three CD34+ cell subsets described above were identified in every NBM while, LP and NP were detected in only 59% and 100% of LR-MDS and in 15% and 85% of HR-MDS respectively. As compared to NBM, in both groups of MDS (LR-MDS and HR-MDS) showed the following aberrations: 1) decreased expression of CD45 on the IM (37% and 55% of the cases, respectively), 2) lack of cyCD79a in LP (60% and 100% of cases, respectively), 3) increased expression of CD117 in CD34+ myeloid precursors, and decreased CD33 expression in NP. In addition, in LR-MDS decreased expression of CD45 in the LP (50%), as well as decreased numbers of NP expressing CD33 (42%) and increased numbers of CD7+ NP (37% of cases), were detected. In turn, HR-MDS patients, but not LR-MDS cases, showed increased numbers of IP (33% of the cases) and low percentages of NP (56% of the cases), decreased expression of cyMPO in NP (41% of the cases) and of CD33 in both the NP (42% of the cases) and IP (36% of the cases) together with increased expression of CD117 and CD13 in both the IP (67% and 39%, respectively) and NP (48% and 37% of the patients, respectively); in addition, decreased expression of nTdT in LP (20%) and abnormally low numbers of CD34+cells expressing the CD65, CD64, CD7 and CD123, were also detected in this patients group. *Conclusion.* Our results show the presence of multiple phenotypic alterations in CD34+ BM myeloid and lymphoid precursors in patients with MDS, these abnormalities being more pronounced in HR-MDS as compared to LR cases.

0463

FUNCTIONAL TLR-4 IN MDS BONE MARROW CELLS: INVOLVEMENT OF TNFA MEDIATED TLR-4 EXPRESSION IN INCREASED APOPTOSIS

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Background. In Myelodysplastic syndromes (MDS), TNFa over-expression is implicated in abnormal gene expression resulting in ineffective hematopoiesis and increased apoptotic death, suggesting the therapeutic potential of TNFa suppression. Toll-like receptors (TLRs) are members of a conserved family of type I transmembrane receptors characterised by an intracellular signalling domain homologue to the IL-1R. TLR expression is induced, by TNFα and, as shown by recent studies, over-expression of TLR-4 leads to increased apoptosis through a Fas-associated pathway. *Aims.* In view of the excessive production of TNFa in MDS and its potential to induce TLR expression as well as the TLR-mediated apoptotic pathway, we reasoned that measurement of TLR expression levels and their functional ability in MDS cells, could provide insight to the MDS elevated apoptosis. Furthermore we examined whether anti-TNFa or anti-TLR-4 treatment affects TLR-4 expression levels and apoptosis. *Methods.* BMMC and CD34+ cells from 20 controls and 12 MDS patients were included in the study. THP-1 cells served as positive control for the TLR expression. All cell lines studied were treated for 12, 24 and 48 hours, with LPS (TLR-4 ligand, 1 μg/mL), TNFa (200-IU/mL), anti-TNFα (infliximab; 10 μg/mL), anti-TLR-4 (10 μg/mL) and simultaneous LPS and TNFα blockage. Quantitative Real-time PCR assessed TLR-1,-2,-3 and -4 mRNA levels. TLR-4 and ICAM.1 (positive triggering of TLRs) protein expression was examined by flow cytometry. Apoptosis was monitored by Annexin-V binding assay. *Results.* Comparison of the expression levels of TLR-1,-2,-3 and 4 mRNA between MDS and controls revealed that MDS-BMMC displayed significant higher levels of TLR-1 (p<0.005), TLR-2 (p<0.05) and TLR-4 (p<0.001) mRNA while MDS-CD34+ cells showed statistically significant higher levels of TLR-4 (p<0.005) mRNA. These results reveal an over-expression of TLR-4 in both the progenitor and differentiated cells. TNFa treatment resulted to a 70.0±2.0% increase of the TLR-4 and 20-fold increase of ICAM.1 protein expression. LPS treatment led to TLR-4 induction at both the mRNA (2-fold) and protein level (5 and 1.5 fold) and strongly induced ICAM.1 expression (80-fold). Anti-TNFa treatment completely inhibited the TLR-4 protein expression in resting and in LPS-stimulated BMMC and THP-1 cells, whereas an 80% reduction was observed at the mRNA level. TNFa and anti-TNFa modulations revealed that both the constitutional and the induced TLR-4 expression is TNFα mediated. Double staining revealed that 73.0±6% of Annexin-V+ cells were TLR-4+, while 83.0±6% of TLR-4+ cells were Annexin-V positive in untreated BMMC, indicating a positive correlation between apoptosis and TLR-4 expression. Apoptosis increased after TNFa treatment or LPS-stimulation in both BMMC and THP-1 (resting: 10.0±2.7% and 12.2±0.8%, TNFa: 19.0±1.3% and 16.0±0.7%, LPS: 27.0±2.9% and 42±1.4% respectively). Anti-TNFa and anti-TLR-4 treatment reduced apoptosis in LPS-treated cells (20% and 26%, respectively). *Summary/Conclusion.* TLR-4 is over-expressed in MDS derived BMMC and CD34+ cells. Both the constitutional and the LPS-induced TLR-4 expression, is TNFa dependent. The majority (73%) of the apoptotic MDS cells are TLR-4+. TLR-4 triggering leads to a strong induction of apoptosis that only to a percentage is TNFα mediated. Concluding, MDS patients over-express TNFa mediated functional TLR-4, which is implicated in both TNF-depended and independed apoptosis.

0464

ABNORMALITIES OF BONE MARROW MESENCHYMAL STEM CELLS IN PATIENTS WITH MYELODYSPLASTIC SYNDROMES

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Background. Patients with Myelodysplastic Syndromes (MDS) display frequently abnormalities of the bone marrow (BM) microenvironmental cells in terms of increased production of inflammatory cytokines and defective support of haemopoiesis. Whether, however, there is a primary defect at the mesechymal stem cell (MSC) level in these patients remains unknown. *Aims.* To study the reserves, the functional characteristics and the differentiation potential of BM MSCs in patients with MDS. *Methods.* Fifteen patients with MDS [4 with Refractory Anaemia (RA) and 11 with RA with excess of blasts (RAEB)] and 22 age- and sex-matched healthy controls were studied after informed consent. The BM mononuclear cells (BMMCs) were isolated from posterior iliac crest aspirates and the MSCs were expanded according to a standard protocol. MSCs were characterized by their immunophenotypic characteristics (CD45-,CD14-,CD34-, CD90+,CD73+,CD44+,CD29+, CD105+, CD146+) and their adipogenic (Oil red O stain and aP2 and PPAR-γ expression by RT-PCR), osteogenic (ALP/Von Kossa stain and ALP and CBFA1 expression by RT-PCR), and chondrogenic (Masson and Alcian blue stain and Collagen II and Aggrecan expression by RT-PCR) potential after induction of differentiation in appropriate media.The frequency of MSCs in the BMMC fraction was evaluated by means of a limiting-dilution assay (LDA) based on the Poisson probability. The functional characteristics of MSCs were studied by evaluating (a) their clonogenic potential using a standard colony forming unit-fibroblast (CFU-F) assay and enumerating the CFU-Fs/100MSCs plated through passages (P), (b) their proliferative potential time-course by using the MTT assay and evaluating the cell doubling time (2^n=cells counted/cells plated) in each passage. *Results.* MDS patients displayed normal number (18.32±13.56 MSCs/10^5 BMMCs in the patients versus 23.78±16.49 MSCs/10^5 BMMCs in the controls; p=0.423) and normal immunophenotypic characteristics of BM MSCs. The chondrogenic, osteogenic and adipogenic potential of patient MSCs did not differ from the respective of the controls as was evaluated by the collagen II and aggrecan, the ALP and CBFA1, and the aP2 and PPAR-γ mRNA expression, respectively, by means of a semi-quantitative RT-PCR. Compared to healthy controls, however, patient MSCs displayed impaired CFU-F potential time-course (p<0.001; P1-P6) as well as impaired proliferative capacity. This was demonstrated by the MTT assay (p< 0.01 at P1) and the cell doubling time time-course (p<0.001; P1-P7). *Summary-Conclusions.* Patients with MDS display normal number and differentiation potential of BM MSCs. The clonogenic and proloferative potential of patient MSCs, however, is defective compared to the respective of the healthy controls. Analysis of the production of growth factors and inhibitors at protein level, evaluation of the telomeric length as well as cytogenetic analysis of patient MSCs is currently under investigation to elucidate further the pathophysiologic basis of the observed MSC abnormalities in MDS patients.

SIMULTANEOUS SESSIONS

Chronic myeloid leukemia

0465

A RANDOMIZED STUDY OF DASATINIB VERSUS ESCALATED DOSE OF IMATINIB IN PATIENTS WITH CHRONIC PHASE CHRONIC MYELOID LEUKEMI RESISTANT TO IMATINIB RESULTS OF CA180017 START-R STUDY

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Background. Patients (pts) who are resistant to IM have few therapeutic options. Escalated dose of IM (800 mg/day) appears to retain some activity. Dasatinib (D) (BMS-354825) is a novel, oral, multi-targeted kinase inhibitor of BCR-ABL and SRC with activity against 18/19 BCR-ABL mutants. *Aim.* To demonstrate the activity of Dasatinib in pts with CP-CML who are resistant to conventional doses of IM. *Methods.* START-R is a multicenter randomized (2:1 ratio) trial of D 70 mg twice daily (BID) and IM 800 mg/day in pts with CP-CML resistant to prior IM 400 to 600 mg/day. Cross-over was allowed for lack of response or intolerance (grade 3-4 non hematologic toxicity). D dose escalation to 90 mg BID was allowed for inadequate response at 12 wks, and dose reduction to 50 or 40 mg BID for drug toxicity. Dose reduction to 600 mg/day was allowed for IM. Evaluations consisted of weekly blood counts for the first 12 wks, bone marrow and cytogenetics every 3 months, molecular monitoring of BCR-ABL transcript levels by real-time quantitative polymerase chain reaction (RT-PCR) every 4 wks for the first 12 wks, and then every 12 wks, and mutation status at baseline and end of treatment. *Results.* From February 2005 to November 2005, a total of 150 pts were randomized (101 to D and 49 to IM). There were 75 (50%) males; median age was 51 yrs (range 24'85). Median time from diagnosis was 59 months and 34% had IM resistant mutations. Prior therapy included interferon in 107 (71%) pts, chemotherapy in 57 (38%) and stem cell transplant in 9 (6%). All pts received prior IM: 96 (64%) had 600 mg/day, 60 (40%) were treated >3 years and 42 (28%) achieved major cytogenetic response (MCyR). An interim analysis was conducted on the first 36 randomized pts (22 D and 14 IM). Confirmed complete hematologic response was documented in 21 D (96%) pts and 13 (93%) IM pts. The MCyR rate at 12 wks was 45% for D and 21% for IM with 7 complete CyR on D and 1 on IM. Best MCyR rate at any time was 11/22 (10 complete) for D and 3/14 (1 complete) for IM. Two (9%) D and 12 (86%) IM pts crossed over. Among pts who crossed to the alternate treatment, best MCyR rate was 6/12 (3 complete) for D and 0/2 for IM. Grade 3-4 neutropenia or thrombopenia were reported in 11 and 9 D pts and 8 and 2 IM pts. Most common grade 1-2 non-hematologic toxicities in D and IM groups were diarrhea (11 and 2), nausea/vomiting (11 and 10), edema (9 and 8), and pleural effusion (3 and 0) *Conclusion.* Dasatinib was effective in pts with CP-CML resistant to IM 400 to 600 mg/day. An updated analysis on all 150 patients will be presented including molecular response.

0466

LONG-TERM BENEFITS OF IMATINIB FOR PATIENTS NEWLY DIAGNOSED WITH CHRONIC MYELOGENOUS LEUKEMIA IN CHRONIC PHASE: THE 5-YEAR UPDATE FROM THE IRIS STUDY

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Background. IM was proven to be superior to IFN+Ara-C for newly diagnosed patients (pts) with CML-CP (O'Brien *et al.,* NEJM 2003). 1106 pts were randomized between June 2000 and Jan 2001 to either IM 400 mg or IFN+Ara-C with 553 pts to each treatment. This abstract is based on data collected up to 54 months after last patient had been recruited on IM. 60 months (5-year) data will be available for presentation. *Methods.* Evaluations included complete hematologic response (CHR), complete/partial cytogenetic response (CCyR/PCyR - defined as 0% / 1-35% Ph+ metaphases respectively), major cytogenetic response (MCyR=CCyR+PCyR), major molecular response (MMR) ' defined as ≥ 3 log reduction of BCR-ABL transcript levels from the standardized baseline, time to progression - defined as loss of CHR/MCyR, evolution to accelerated phase/blast crisis (AP/BC), or death due to any cause during treatment, and overall survival. *Results.* With a median follow-up of 54-months, 72% of the 553 randomized pts remain on initial IM treatment (5% of pts discontinued due to adverse events, 9.5% due to unsatisfactory therapeutic effect and 11% due to other reasons; another 2.5% crossed over to IFN+Ara-C). Overall, the cumulative best response rates of CHR, MCyR and CCyR are 97%, 88% and 82%, respectively. The overall estimated survival was 90% (93% when censored at bone marrow transplant). An estimated 84% of pts have not progressed on treatment and 93% of pts were free from progression to AP/BC. The annual rate of progression to AP/BC of <1% in the fourth year was lower than each of the first three years (1.5, 2.8, 1.6% respectively). Of the pts with MCyR at 12 months (n=436), an estimated 96% were free of progression to AP/BC at 54 months whereas it was only 81% for the 73 pts who did not achieve a MCyR at 12 months (p<0.001). No patient with a MMR at 12 months progressed to AP/BC within 54 months. *Conclusions.* This analysis confirms the high rates and durability of responses to IM. Encouragingly, the rate of progression in the fourth year was lower than in each of the preceding three years. Results further demonstrate the beneficial effect of cytogenetic and molecular responses on long-term outcomes.

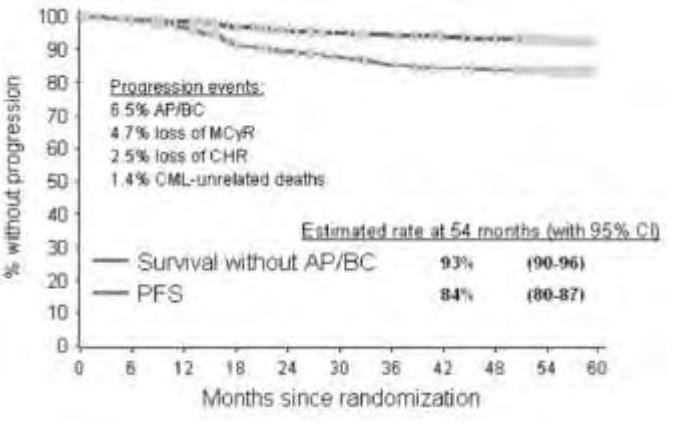

Figure 1. Progression-free survival and survival without AP/BC on first-line imatinib.

0467

A PHASE II STUDY OF DASATINIB IN PATIENTS WITH CHRONIC PHASE CHRONIC MYELOID LEUKEMIA WHO ARE RESISTANT OR INTOLERANT TO IMATINIB: RESULTS OF THE CA180013 START-C STUDY

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Background. Dasatinib (BMS-354825) is a novel, oral, multi-targeted kinase inhibitor of BCR-ABL and SRC kinases with preclinical and clinical activity against imatinib resistant BCR-ABL mutations. *Aims.* To demonstrate the activity of dasatinib in patients (pts) with CP-CML who are resistant to (primary or acquired resistance, or detection of BCR-ABL mutations highly associated with imatinib resistance) or intolerant (grade 3-4 non hematologic or persistant hematologic toxicity) of imatinib. *Methods.* START-C is an open-label Phase II study of dasatinib in imatinib-resistant (IM-R) or intolerant (IM-I) pts with CP-CML. Between February-August 2005, 387 pts were recruited at 75 centers worldwide. Dasatinib was given at 70 mg twice daily (BID) with dose escalation to 90 mg BID in pts lacking response, and dose reductions to 50 and 40 mg BID for intolerance. Evaluations were weekly blood counts

for the first 12 weeks; bone marrow cytology and cytogenetics every 3 months; and molecular monitoring of BCR-ABL transcript levels by real-time quantitative polymerase chain reaction (RT-PCR) every 4 weeks for the first 12 weeks, and then every 12 weeks while on study. The primary endpoint was major cytogenetic response (MCyR) rate. *Results.* Of the 387 pts, 271 were IM-R and 116 were IM-I pts. Median age was 58 yrs (range 21-85); 49% were male. Median time from diagnosis of CML was 61 months. Prior treatment included interferon in 252 (65%) and stem cell transplant in 38 (9.8%). All patients received prior IM: doses >600 mg/day in 214 (55%), >3 years in 207 cases (53%); 141 pts (36%) achieved MCyR on prior IM. Efficacy and safety data are currently available from 186 pts (127 IM-R, 59 IM-I) accrued prior to May 12, 2005. With ≥6 months of follow up, 168 (90%) pts had a complete hematologic response (CHR), and 83 (45%) pts had a MCyR: 40 (31%) of IM-R pts, and 43 (73%) of IM-I pts. Rate of MCyR was 37% among the 65 pts with BCR-ABL mutations. Grade 3/4 neutropenia or thrombocytopenia was reported in 83 (45%) pts and 85 (46%) pts with onset after 4-8 weeks of therapy in most pts. Dose interruptions occurred in 146 (78%), and dose reductions in 96 (52%) pts with an average daily dose of 108 (range 19-169) mg. Non-hematologic toxicity consisted mainly of Grade 1/2 diarrhea, headache, superficial edema, and pleural effusion, with ≤2% Grade 3/4. There was no cross-intolerance between dasatinib and IM. *Conclusions.* Dasatinib demonstrated substantial hematologic and cytogenetic activity in IM-R and IM-I pts with CP-CML. An updated analysis of 387 pts with 6 months of follow up, in addition to the molecular response analysis, will be presented.

0468

RESPONSE TO DASATINIB AFTER IMATINIB FAILURE ACCORDING TO TYPE OF PREEXISTING BCR-ABL MUTATIONS

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Background. Dasatinib (BMS-354825) is a novel, oral, multi-targeted kinase inhibitor of BCR-ABL and SRC kinases with preclinical activity against 19/20 imatinib resistant BCR-ABL mutations and clinical phase I/II efficacy in patients with chronic myelogenous leukemia (CML) and BCR-ABL positive acute lymphoblastic leukemia (ALL). *Aims.* We sought to establish a relationship between type of preexisting BCR-ABL mutations associated with imatinib resistance and efficacy of dasatinib in patients (pts) with CML and ALL. *Methods.* We have investigated peripheral blood samples from 306 pts (166 m, 140 f; median age 60 yrs, range 16-84) who had been enrolled in international phase II studies investigating the activity of 70mg dasatinib BID after imatinib failure (chronic phase, CP, n=123; accelerated phase, AP, n=78; myeloid blast crisis, mBC, n=52; lymphoid blast crisis, lyBC, or ALL, n=53). Screening for BCR-ABL mutations was performed by D-HPLC combined with DNA sequencing. The analysis covered amino acids 207-517 of the BCR-ABL tyrosine kinase domain. Hematologic and cytogenetic response data have been collected sequentially for 6 months after start of dasatinib treatment according to standard definitions. *Results.* Prior to dasatinib, 43 different BCR-ABL mutations involving 34 amino acids were detected in 153/306 pts (50%). 114 pts showed one, 31 pts two, 5 pts three, 2 pts four, and 1 pt five mutations. Mutations were observed in 52 pts in CP (42%), 44 pts in AP (56%), 20 pts in myBC (38%), and 37 pts in lyBC and ALL (70%). There was no specific pattern of the type of mutation in different phases of the disease. In pts with mutations, the overall rate of hematologic response was 67% (90% in CP, 75% in AP, 40% in myBC, and 38% in lyBC/ALL) and was not significantly different for pts with mutations in the P-loop (50/78, 64%), activation loop (19/26, 73%), or other sites (46/77, 60%), except T315I (0/13, 0%). In contrast, major cytogenetic response rates (MCR, <35% Ph^+) within 6 months of therapy differ according to the site of the mutation: Overall (76/194, 39%), P-loop mutations (26/78, 33%), activation loop mutations (15/26, 58%), other mutations (35/77, 45%), T315I mutations (0/13, 0%). Response was delayed in case of E255K/V mutations (HR 7/16, 44%; MCR 13%), whereas F311I/L/V and D276G (n=6/3, HR 100%, MCR 100%) were associated with excellent response to dasatinib therapy. *Conclusions.* Dasatinib is capable to induce hematologic and cytogenetic remissions in a significant proportion of imatinib resistant pts harboring BCR-ABL mutations, except T315I. Response dynamics depend on the individual type of mutation which may be a basis for individual dose adaptation according to the mutation pattern.

0469

PREDICTIVE VALUE OF BCR-ABL TRANSCRIPT LEVELS AFTER 12 MONTHS OF IMATINIB MONOTHERAPY FOR THE OUTCOME OF CML PATIENTS AFTER 5 YEARS

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Background. Despite most chronic myelogenous leukemia (CML) patients (pts) treated with imatinib achieve a complete cytogenetic remission (CCR), about 4% of pts per year will relapse. The achievement of an at least 3 log reduction of BCR-ABL transcript levels compared with a predefined baseline has been associated with a favorable long term outcome. *Aims.* We sought to establish a relationship between 12 month quantitative PCR data and relapse free survival based on the determination of ratios BCR-ABL/ABL. *Methods.* Serial peripheral blood (n=874) and bone marrow (n=685) samples from 68 pts randomized for first line imatinib therapy within the international IRIS trial have been investigated employing qualitative and quantitative RT-PCR and conventional cytogenetics. Degree of molecular response was classified retrospectively using a direct comparison of the log reduction terminology and the ratio BCR-ABL/ABL: Ratios of 0.01%, 0.12%, and 1.4% represent a 4, 3, and 2-log-reduction, respectively. *Results.* After 12 months of imatinib therapy, ratios <0.01% were achieved in 4 pts (cohort 1, 6%); ratios of 0.01-0.12% in 22 cases (cohort 2, 32%); >0.12-1.4% in 27 pts (cohort 3, 40%), and >1.4% in 15 pts (cohort 4, 22%). Overall median observation time was 57 mo and not different between the 4 cohorts. The most recent analysis showed CCR in 4/4 pts in cohort 1 (100%), 21/22 pts in cohort 2 (95%), 24/27 pts (89%) in cohort 3, and 7/15 pts in cohort 4 (47%, p=0.0006). Most recent Q-PCR values differ significantly between cohorts (cohort 1 0.0091%, cohort 2 0.0063%, cohort 3 0.037%, cohort 4 1.1%, p=0.0002). The same applies to overall best Q-PCR results (cohort 1 0.006%, cohort 2 0.00095%, cohort 3 0.026%, cohort 4 1.1%, p<0.0001). In 10/26 (38%) pts of cohorts 1+2 BCR-ABL was not detectable by a sensitive nested PCR at the most recent analysis whereas none of 42 pts demonstrated undetectable BCR-ABL in cohorts 3 and 4 (p<0.0001). Five pts have relapsed with reappearance of Ph+ metaphases after a median of 6 mo (range 3-9) post first CCR. These pts belonged to cohorts 2 (n=1), 3 (n=2), and 4 (n=2) after 12 mo of therapy. There was a trend towards higher ratios BCR-ABL/ABL at mo 12 in pts with subsequent relapse compared to those in continuous CCR (0.40 vs 0.15%, p=0.29). Two pts progressed to accelerated phase/blast crisis after 16 and 54 months, these pts had 70 and 100% Ph+ metaphases at mo 12, respectively. *Conclusions.* BCR-ABL transcript levels after 12 mo of imatinib therapy are predictive for long term cytogenetic and molecular response. Overall rate of CCR parallels the degree of early molecular response. A ratio BCR-ABL/ABL <0.12% is predictive for a better chance to achieve CCR accompanied by a >95% probability of CCR as well as a 38% chance of becoming nested PCR negative after 5 years. The *in vivo* data confirm the equivalence of the definition of a major molecular response as a 3-log reduction compared to a predefined baseline vs the ratio BCR-ABL/ABL of 0.12%.

Acute myeloid leukemia

0470

DOSE-DEPENDENT COMPETITION BETWEEN NPM LEUKEMIC MUTANTS AND ARF PROTEIN FOR SUBCELLULAR DISTRIBUTION: A NUCLEAR TUG-OF-WAR

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Acute myeloid leukemia (AML) is frequently targeted by mutations at exon-12 of the nucleophosmin (NPM) gene (Falini *et al.*, NEJM, 352:254, 2005), which 1) disrupt either tryptophan 290 or trytophans 288 and 290, constituting the nucleolar localization signal (NoLS); and 2) create a new carboxy-terminal Nuclear Export Signal (NES) motif, with 6 variations observed to date. Both abnormalities underlie aberrant NPM accumulation in leukemic cell cytoplasm (NPMc+ AML). In NPM mutants, the new NES motif non-randomly correlates with NoLS disruption at the C-terminus. The most common NES motif (LxxxVxxVxL) always associates with mutations of tryptophans(W) 288 and 290, e.g. mutant A; a NES variant sequence, such as *LxxxLxxVxL*, always associates with W288 retention in rare NPM mutants, e.g. mutant E (Falini *et al.*, Blood, pub-ahead, February 2, 2006). These findings suggest diverse sequences of mutant NES motifs function differently. Mutated NPM dislocates Arf from nucleoli, shortens its half-life and blunts its functions (Den Besten W *et al.*, Cell Cycle, 4:1593, 2006). In different NPM mutants this study addressed: i) the role of variations in NES motifs; and ii) interactions with Arf. i) Role of different NES motifs in altered NPM nucleo-cytoplasmic traffic: Arf-negative NIH-3T3 cells were transfected with eGFP-tagged NPM mutant A in which W288 had been artificially inserted by site-directed mutagenesis (eGFP-NPMmA_C288W). Unlike mutant E, which is unaffected by the presence of W288, this protein displayed greatly reduced cytoplasmic export. Additionally, in NPM mutant E, replacing the *LxxxLxxVxL* NES sequence with *LxxxVxxVxL* (eGFP-NPMmE_LVVL) partially relocated mutant E to the nucleus. These results demonstrate efficiency differences between NES: *LxxxVxxVxL* is weaker than *LxxxLxxVxL*. The former is strong enough to export the NPM mutants only if both tryptophans are mutated whilst *LxxxLxxVxL* is needed if W288 is retained. ii) Interaction of NPM mutants with Arf: We investigated how changes at the NPM mutant C-terminus influence NPM-Arf binding, and NPM and Arf subcellular distribution. Arf-negative NIH-3T3 cells were co-transfected with DsRed-tagged Arf (DsRed-monomer-Arf) and eGFP-tagged NPM mutants. Arf partially relocated NPM mutants A and E from cytoplasm to nucleus in a dose-related manner. In turn, NPM mutants partially relocated Arf from the nucleolus to nucleoplasm and cytoplasm. These results demonstrate a reciprocal interaction between Arf and NPM mutants. Moreover lower doses of Arf completely relocated artificial mutants eGFP-NPMmA_C288W and eGFP-NPMmE_LVVL to the nucleus, suggesting these artificial mutants have a stronger affinity for Arf than NPM mutants A and E. Co-immunoprecipitation studies showed mutants A and E bind less Arf than wild-type NPM or eGFP-NPMmA_C288W and eGFP-NPMmE_LVVL. *Conclusions.* The non-random correlation between NPM NoLS disruption and NES sequence variants is feasibly explained by need for 1) efficient cytoplasmic accumulation of mutated NPM, and 2) less efficient binding of mutant NPM to Arf as compared to wild-type NPM. Both mechanisms may contribute to Arf dislocation/degradation, thus having the same functional consequences as NPM silencing. These findings may be relevant to the pathogenesis of NPMc+ AML.

0471

A NOVEL MOLECULAR MECHANISM LEADING TO PRIMARY RESISTANCE TO FLT3-TYROSINE KINASE INHIBITORS IN AML BY FORMATION OF FLT3-ITD627E

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Background. Activating mutations in the FLT3 receptor tyrosine kinase are detected in approximately 35% of AML patients. Currently, several small molecule FLT3 tyrosine kinase inhibitors (TKI) are being tested in clinical trials. These studies showed that monotherapy using FLT3-TKI results in measurable clinical responses including significant reductions in PB and BM blasts. Most of these responses are transient, however, in a subset of patients blast recurrence is preceded by an interval of prolonged remission. Currently, the etiology of primary and secondary clinical resistance to FLT3-TKI in AML is poorly understood but is of major significance for the development of future therapeutic strategies using these compounds in combination with chemotherapy. Recently, we described mechanisms of secondary resistance toward PKC412-treatment in a subset of AML patients relapsing after various durations of remission and identified a resistance mutation (N676K) in the tyrosine kinase domain of FLT3 (Heidel *et al.*, 2006). *Aims.* Here, we investigate molecular mechanisms leading to primary clinical resistance toward PKC412-therapy. *Methods.* In AML patients showing primary resistance to monotherapy with PKC412 within a clinical phase II study, molecular analysis of clinical material was performed. In addition, 753 unselected AML cases were screened for genomic localization of ITD integration sites. *Results.* Ex-vivo analysis of primary AML-blasts at the timepoint of clinical resistance to PKC412 in an index AML patient showed persistent tyrosine phosphorylation of FLT3 despite sufficient PKC412-plasma levels. FLT3 sequence analysis revealed an ITD-allele integrating in exon 15 at codon 627 and thereby leading to an aa-exchange at codon 627 (A627E). This particular position has previously been implicated in FLT3 TKI-resistance in *in vitro* models (Cools *et al.*, 2004). Cloning and expression of this ITD-allele (FLT3-ITD627E) in 32D cells led to IL-3 independent growth as well as inherent high level resistance toward PKC412-treatment in apoptosis assays. Moreover, FLT3-ITD627E expressing 32D cells were also resistant toward treatment with alternative FLT3-TKIs (SU5614 and K-252a (similar to CEP-701)). This demonstrates that the ITD627E allele is sufficient to confer resistance to these FLT3-TKIs. Interestingly, additional analysis revealed that this particular ITD-allele was already present before start of treatment with PKC412. Thus, this well explains the clinical course of the patient being refractory to PKC412-therapy. To investigate the hypothesis, that integration of ITDs in exon 15 of FLT3 may be generally associated with primary resistance to FLT3-TKI, experiments are currently underway, and will be presented, to determine the relevance of the position of ITD-integration for resistance towards FLT3-TKIs. Specifically, we are investigating the role of ITD integration at codon 627 without mutation of aa A627. To estimate the prevalence of ITD integration in exon 15 and in codon 627, in particular, we screened FLT3-ITD sequences of 753 unselected AML cases using samples collected at initial diagnosis (de-novo AML, secondary AML after MDS, chemotherapy-related AML). This analysis showed that 35 (4.6%) of these patients had ITDs integrating in exon 15 but only 1 additional patient with ITD integration in codon 627 was identified. *Conclusions.* Our results present evidence for a novel molecular mechanism leading to primary resistance to FLT3-TKIs. However, analysis of a large cohort of AML cases suggests that this is a rare event and does not represent a therapeutic obstacle for FLT3-TKIs in the vast majority of patients carrying FLT3-ITDs.

0472

MICROARRAY-BASED CHARACTERIZATION OF AML WITH COMPLEX KARYOTYPES DISCLOSE NOVEL GENOMIC IMBALANCES HARBORING NEW CANDIDATE GENES

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Approximately 10 to 15% of acute myeloid leukemia (AML) cases exhibit complex karyotypes, i.e., three or more chromosome abnormalities without presence of a specific fusion transcript. To identify novel genomic regions of interest in this AML subgroup we applied comparative genomic hybridization to microarrays (array-CGH) allowing high-resolution genome-wide screening of genomic imbalances. Therefore, we designed a 2.8k-microarray consisting of 2799 different BAC- or PAC-vectors with an average resolution of approximately 2 Mb. Using this microarray platform, 83 AML cases with complex karyotypes were analyzed. Genomic losses were found more frequently than gains; the most frequent losses were deletions of 5q (71%), 17p (53%), 7q (48%); followed by deletions of 18q (30%), 16q (28%), 3p and 12q (20% each), 12p (18%), 20q (17%), and 11q (12%). The most frequent genomic gains were trisomies of 11q (39%) and 8q (31%); followed by trisomies of 1p (22%), 21q (20%), 9p (14%), 22q (13%), 13q (12%), and 6p (10%). In part, some critical segments were delineated to genomic fragments of 0.8 to a few megabase pairs in size. Furthermore, 47 high-level DNA amplifications in 19 different regions were identified; amplifications occurring in at least two cases mapped to (candidate genes in the amplicon) 11q23.3-q24.1 (n=10; ETS, FLI1); 11q23.3 (n=8; MLL, DDX6); 21q22 (n=5; ERG, ETS2); 13q12 (n=3; CDX2, FLT1, FLT3, PAN3); 8q24 (n=3; C8FW, MYC); 9p24 (n=2; JAK2); 12p13 (n=2; FGF6, CCND2); and 20q11 (n=2; ID1, BCL2L1). For better characterization of the amplicons, we applied array-CGH using a 6.0k-microarray with an average resolution of approximately 1 Mb revealing highly complex amplicon structures. Furthermore, in a subset of cases we profiled global gene expression detecting a gene dosage effect with significant lower/higher average gene expression levels across the genes located in the lost/gained regions as compared to unaltered cases. Additionally, parallel analysis displayed overexpressed candidate genes in critical amplified region, e.g., C8FW and MYC in 8q24 as well as FLT3 and CDX2 in 13q12. In conclusion, using high-resolution genome-wide screening tools such as array-CGH allows to unravel the enormous genetic diversity of AML cases with complex karyotypes, and correlation with global gene expression studies facilitates the delineation of disease-related candidate genes located in the critical regions.

0473

GENE EXPRESSION BASED CHARACTERIZATION OF NPM1-MUTATED/FLT3 ITD-NEGATIVE ACUTE MYELOID LEUKEMIA WITH NORMAL KARYOTYPE

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Background. Acute myeloid leukemia (AML) with normal karyotype encompasses a large number of molecularly distinct variants. While the presence of internal tandem duplications (ITDs) of the FLT3 (fms-related tyrosine kinase 3) gene is associated with poor outcome, recently mutations of the NPM1 (nucleophosmin) gene have been shown to be prognostically favorable. However, this effect is mainly attributed to the NPM1-mutated/FLT3 ITD-negative AML cases. While NPM1-mutated cases are characterized by a distinct gene expression pattern, it remains unclear whether NPM1-mutated/FLT3 ITD-negative cases also display a characteristic signature, which might provide additional insights into the molecular basis for the good clinical outcome. *Aims.* Having demonstrated the presence of a signature correlated with NPM1-mutational status, we sought to define a molecular profile for AML cases with NPM1-mutated/FLT3 ITD-negative normal karyotype disease. *Methods.* Towards this goal, we have profiled gene expression of 138 samples of adult AML patients with normal karyotype using DNA microarray technology. All samples analyzed were derived from AML patients entered within the randomized multicenter treatment trial HD-98A of the German-Austrian AML Study Group (AMLSG). *Results.* Based on supervised data analysis using SAM (Significance analysis of Microarrays), we were able to identify a 116-genes comprising expression pattern correlated with NPM1-mutated and FLT3 ITD-negative AML cases. In accordance with previous findings in NPM1-mutated cases (Alcalay *et al.* 2005, Verhaak *et al.* 2005), the NPM1-mutated/FLT3 ITD-negative pattern was also in part characterized by a prominent HOX gene cluster, which clearly separated the NPM1-wildtype from the NPM1-mutated cases. Similarly, the expression levels of BAALC and MN1 showed a correlation with the NPM1 mutational status, with NPM1-unmutated cases displaying higher expression in our data set. However, as expected the newly defined signature also defined a NPM1-mutated group that did not contain many FLT3 ITD-positive samples. This group was characterized by several interesting genes including for example TLE1, which encodes a Groucho/TLE family protein. Groucho/TLE family proteins are transcriptional co-repressors, which mediate repression essential in embryonic development and are involved in regulation of Wnt signaling in adult tissue. Moreover, we identified several other genes of potential pathogenic relevance which also have been previously shown to be predictive in normal karyotype AML. *Conclusions.* Our findings support a distinct molecular mechanism associated with the favorable outcome of NPM1-mutated/FLT3 ITD-negative AML cases. Furthermore, the reported signature might contribute to improved risk stratification and clinical management of AML patients with normal karyotype disease.

0474

EFFECT OF MDR1 SINGLE NUCLEOTIDE POLYMORPHISMS (SNPS) C1236T, G2677T AND C3435T ON MDR1 FUNCTION AND EXPRESSION IN LEUKEMIC BLASTS, AND ON TREATMENT OUTCOME IN ELDERLY PATIENTS WITH AML

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Background. The classical multidrug resistance (MDR) gene MDR1 (ABCB1) encodes for the drug efflux pump P-glycoprotein (P-gp). MDR1 expression is an adverse prognostic factor for treatment outcome in acute myeloid leukemia (AML), and is more frequently observed in older patients. Single nucleotide polymorphisms (SNPs) of the MDR1 gene, C1236T, G2677T and C3435T, have been associated with altered drug metabolism and treatment outcome. *Aims.* We prospectively determined these SNPs in a cohort of patients with AML of 60 years and older, and evaluated their relevance for MDR1 function and expression, MDR1 mRNA expression and clinical outcome. *Methods.* We have analyzed purified bone marrow derived leukemic blasts of 150 patients treated within the multicenter, randomized phase 3 trial HOVON 31 AML (Novartis PSC C 302-E-00) (Van der Holt *et al.* Blood. 2005;106:2646-2654). In that trial, 419 eligible Caucasian patients aged 60 years and older with previously untreated *de novo* and secondary AML (FAB classification M0-M2 and M4-M7) were randomized to receive standard induction chemotherapy with or without the MDR1 inhibitor PSC-833 (Valspodar®, Amdray®, Novartis Pharmaceuticals, Basle, Switzerland). The 150 patients genotyped patients were selected for MDR1 analysis based on availability of blast samples in our cell bank. The significance of the allelic MDR1 variants of C1236T, G2677T and C3435T was evaluated with respect to P-gp expression and function in leukemic blasts, and MDR1 mRNA expression levels. The relationship between each of these genetic polymorphisms of MDR1 with clinical outcome, i.e. complete response (CR) rate, event-free survival (EFS), disease free survival (DFS) and overall survival (OS) was also assessed.

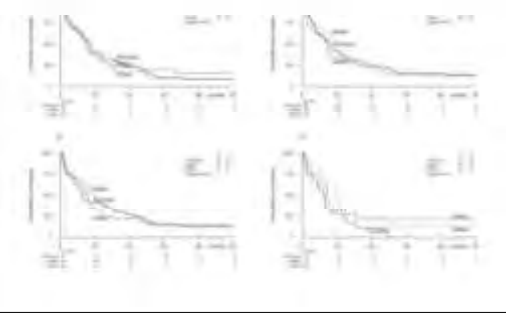

Figure 1. OS of elderly AML patients, by genotype. (A) C1236T. (B) G2677T. (C) C3435T. (D) Patients with the same variant for the 3 SNPs.

Results. Each of the 3 SNPs was in Hardy-Weinberg disequilibrium

(p<0.001), contrary to other published results. Each combination of two SNPs was in linkage disequilibrium (p<0.001), which confirms results reported by Illmer et al (Cancer Res. 2002;62:955-4962). Patient baseline characteristics were not significantly different between wild-type, heterozygous or homozygous mutant patients, neither for the 3 genetic polymorphisms, nor for the patients with the same allelic variant of all 3 SNPs. P-gp efflux and expression data in purified AML blasts and in the CD34-positive subpopulation, as well as the MDR1 mRNA expression levels of AML patients did not vary significantly among any of the allelic variants of MDR1. All functional and expression data were highly correlated (p<0.001). The median follow up of 24 patients still alive was 57 months (range, 8-81). No statistically significant differences in CR rate and survival endpoints were observed between the allelic subgroups (Figure 1), neither unadjusted nor adjusted for treatment arm, nor was there any apparent interaction between the allelic variants of each SNP and treatment arm with respect to outcome. *Summary/Conclusions.* In AML patients aged 60+, allelic MDR1 variations of C1236T, G2677T or C3435T are not associated with altered MDR1 function, nor with MDR1 expression at the transcriptional or translational level in leukemic blasts, and they do not significantly impact on clinical prognosis, suggesting that they do not exert a major impact on drug resistance in elderly patients with AML.

Stem cell biology

0475

THE MOLECULAR SIGNATURE OF PURIFIED CANCER STEM CELLS REVEALS A STEM CELL ORIGIN OF 5Q- SYNDROME

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Background. Although it has been postulated that leukemic and other cancer stem cells frequently may originate in the corresponding rare multipotent stem cell population, conclusive evidence for such a model has only been obtained for Philadelphia chromosome-positive chronic myeloid leukemia. *Aim.* To identify the origin of cancer stem cells by applying global gene expression profiling and to uncover MDS stem cell specific gene expressions. *Methods.* Fluorescence activated cell sorting (FACS) for enrichment of candidate MDS 5q- syndrome stem cells, fluorescence *in situ* hybridization (FISH) to show clonal (5q-) involvement, long-term culture initiating-cell (LTC-IC) assay to show stem cell function and oligonucleotide microarrays to evaluate the global gene expression profile of candidate MDS stem cells. *Results.* Global gene expression profiles of candidate MDS 5q- stem cells (CD34+CD38-Thy-1+) and normal stem cells (CD34+CD38-Thy-1+) are more similar than candidate MDS stem cells and normal progenitors (CD34+CD38+Thy-1-) are. However, BMI1 and CEBPalfa are up-regulated in MDS stem cells from most patients. Furthermore, these differences are specific for MDS stem cells since CEBPalfa is down-regulated and BMI1 is unaffected in MDS progenitors. *Conclusions.* Global gene expression profiling supports that MDS 5q- syndrome originates in normal stem cells. BMI1, a critical regulator of self-renewal, is up-regulated in MDS stem cells, as is the myeloid transcription factor CEBPalfa. These changes are specific for MDS stem cells and could potentially be involved in defining the MDS 5q- syndrome stem cell clonal advantage and defective differentiation process. We have demonstrated the importance of identifying the specific cancer stem cell population to uncover potential gene expression changes contributing to unique cancer stem cell properties

0476

PROTEIN KINASE B: A MOLECULAR SWITCH IN REGULATION OF LINEAGE CHOICE DECISIONS DURING MYELOPOIESIS

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Introduction. Hematopoiesis is a highly regulated process resulting in the formation of all blood lineages. The specific signal transduction pathways involved in lineage choices during hematopoiesis remain largely unsolved. The PI3K/PKB pathway has been reported to play a critical role in proliferation and survival of cells, however, a role in regulating hematopoiesis is largely unknown. *Aim.* The aim of this project is to investigate whether the PI3K signaling module plays a role in regulation of myelopoiesis. *Methods.* Human umbilical cord blood derived CD34+ cells cultured in presence of IL-5 or G-CSF resulting in eosinophil or neutrophil differentiation, respectively, were either treated with pharmacological inhibitors to block the PI3K signaling pathway or retrovirally transduced to ectopically express constitutively active PKB, a downstream target of PKB. *Results.* Inhibition of PKB blocked progenitor proliferation without affecting cell survival. Interestingly, inhibition of PKB abrogated neutrophil differentiation, but conversely, dramatically enhanced eosinophil maturation. Retroviral transduction of CD34+ cells with constitutively active PKB (myrPKB) resulted in enhanced neutrophil differentiation and monocyte development, whereas eosinophil differentiation was blocked. In contrast, dominant-negative PKB (PKBcaax) induced eosinophil differentiation and inhibited neutrophil maturation. Transplantation of β2-microglobulin (-/-) NOD/SCID mice with CD34+

cells ectopically expressing myrPKB resulted in enhanced neutrophil and monocyte development, whereas ectopic expression of PKBcaax induced eosinophil development. Inhibitory phosphorylation of C/EBPα, a transcription factor known to play a critical role in regulation of myelopoiesis, was abbrogated upon PKB activation in hematopoietic progenitors. *Conclusion.* These results demonstrate that PKB activity plays a critical role in regulation of cell fate choices during myeloid lineage commitment. High PKB activity promotes neutrophil differentiation and monocyte development, while reduction of PKB activity is required to induce eosinophil differentiation.

0477

CONSTITUTIVE EXPRESSION OF THE 'LYMPHOID ENHANCER FACTOR 1' (LEF-1) PERTURBS HAEMATOPOIETIC DEVELOPMENT AND INDUCES LEUKEMIA IN A SUBSET OF TRANSPLANTED MICE

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Background. Lef-1 is a key transcription factor of the Wnt/β-catenin signalling pathway and is crucially linked to normal B- and T-cell development. Recently its aberrant expression has been associated with different types of leukemia. *Aims.* Aims of this project were to elucidate the expression pattern of Lef-1 in different haematopoietic subpopulations and to test whether constitutive expression of this transcription factor affects early haematopoietic development. *Methods.* Expression Analysis was performed by using semi-quantitative RT-PCR and Real-Time PCR. Functional relevance was demonstrated by induction of constitutive expression of wild type Lef-1 (WT) and of a constitutive active Lef-1 mutant (CA) in primary murine bone marrow cells by retroviral gene transfer, using a MSCV based retroviral construct with an IRES-GFP cassette. *Results.* Analysis of Lef-1 expression showed expression in both lymphoid and myeloid subpopulations, but also in highly purified haematopoietic stem cells. *in vitro*, at the level of clonogenic progenitor cells, the colony forming potential of progenitors was increased more than 2-fold by both WT and CA compared to the empty vector control (n=4; WT: $p<0.02$; CA: $p<0.05$). At the level of the short-term repopulating stem cell, both Lef-1 constructs remarkably increased the number of spleen colonies, resulting in a 4fold and 7fold increase in the CFU-S frequency in WT and CA compared to the GFP control, respectively (median 80 (WT) and 135 (CA) CFU-S/1×10^5 cells versus 20 CFU-S/1×10^5 cells, respectively; $p<0.001$; WT n=7, CA n=6, control n=19). To assess the impact of Lef-1 on long-term repopulating stem cells mice were transplanted with BM cells transduced either with WT Lef-1 or CA-Lef-1. *in vivo*, normal haematopoietic development was severely perturbed in transplanted mice. Both constructs induced a reduction of lymphoid cells as well as a dramatic increase of myeloid cells with an inversion of the lymphoid/myeloid ratio (WT: ratio 0.43, $p<0.01$; CA: ratio 0.10, $p<0.001$; vs. 1.02 in control mice). Engrafted mice succumbed to a lethal myeloproliferative syndrome or to acute leukemias, which were readily transplantable into secondary recipients and showed indefinite IL-3 dependent cell growth *in vitro*. *Conclusions.* These data show that balanced expression of Lef-1 plays a key role in early haematopoietic development and that deregulation of this transcription factor favours the development of myeloid malignancies.

0478

ALTERED PROLIFERATION/DIFFERENTIATION POTENTIAL OF COMMON MEGAKARYOCYTIC-ERYTHROID PROGENITORS FROM MICE CARRYNG THE HYPOMORPHIC GATA1 LOW MUTATION

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Background. Several recent evidences suggest that, in addition to its function at late stages of maturation, Gata1 also controls the proliferation/differentiation potential of hemopoietic progenitor cells. We have previously shown that the hemopoietic tissues from mice carrying the hypomorphic Gata1low mutation contain numerous (~10% of all the cells) *unique* tri-lineage progenitor cells, committed toward the erythroid, megakaryocytic and mastocytic lineage (Migliaccio *et al.* J.Exp.Med. 2003.197:281). Although predicted by the stochastic model of hematopoietic commitment, the nature of these cells is unclear because progenitors with such a biological function have not been prospectively isolated from mouse tissues as yet. *Aims.* To clarify the effect of the Gata1low mutation at the level of the hematopoietic progenitor cells by prospectively identifying the tri-lineage progenitors present in the tissues of these mutants. *Methods.* The number and function of mast cells from Gata1low mice, normal littermates (positive controls) and W/Wv mutants (negative controls) was compared in bone marrow derived mast cell cultures (BMMC). The frequency of the common myeloid (CMP), granulocytic-monocytic (GMP), megakaryocyte-erythroid (MEP) and mast cell (MCP) progenitors in the marrow and spleen from wild type and Gata1low littermates was compared on the basis of specific antigenic profiles. The biological functions of these cells was investigated in single cell cultures followed by single cell replating experiments. *Results.* BMMC from Gata1low mice generated differentiation defective mast cells that proliferated 10-fold more than their normal counterparts and consistently gave rise to growth factor dependent tri-lineage (erythroid, megakaryocytic and mastocytic) cell lines. Although the frequency of CMP, GMP and MEP was normal, MCP were not detectable in the tissues from Gata1low mice. On the other hand, MEP isolated from mutant mice, in contrast to those isolated from wild type controls, generated, after seven days of culture, not only erythroblasts and megakaryocytes, but also mast cells and their precursors. Many (40%) of the mutant CMP and MEP generated in culture, at the single cell level, high numbers of cells that could be sequentially recloned for up to 40 days. By this time, >90% of the cells present in the culture, could be replated at the single cell level and their progeny resembled in morphology the cell lines obtained from Gata1low mice in BMMC. *Conclusions.* These results indicate that CMP, GMP and MEP, but not MCP, are present in the tissues of the Gata1low mice. However, in these mutants, the mast cell generating activity is abnormally retained by MEP, that represents the 'unique' tri-lineage progenitor previously identified in the tissues from these mutants. Therefore, Gata1low MEP are antigenically but not functionally, equivalent to MEP from wild-type animals. These results indicate as new target for Gata1low mutation the restriction point when CMP became committed toward MEP or MCP.

0479

CD97 IS DIFFERENTIALLY EXPRESSED ON MURINE HEMATOPOIETIC STEM CELLS AND PROGENITOR CELLS

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CD97 is a member of the EGF-TM7 family of class II seven-span transmembrane receptors and is broadly expressed on hematopoietic cells including lymphocytes, granulocytes, and monocytes. We have recently demonstrated that CD97 is involved in IL-8-induced hematopoietic stem cell (HSC) mobilization (Blood (2003) 102:455a). To determine a possible role of HSC in this process, we studied the expression of CD97 on HSC. Murine HSC are characterized as c-KitPOSThy-1LOLinNEG/LOSca-1POS and isolation of these cells involves a multistep process consisting of lineage depletion and positive selection. We first studied expression levels on murine bone marrow (BM) cells using a CD97 specific monoclonal antibody (clone 1B2) and FACS analysis. Based on CD97 expression levels, BM cells were then sorted into different fractions and further characterized in colony-forming assays, CAFC assays and in an *in vivo* transplantation model. FACS analysis of BM cells showed three major populations i.e. CD97HI, CD97INT and CD97NEG cells (71.5%, 24.4% and 4.4% of total BM cells respectively). Analysis of CFU-GM colony forming capacity of these BM subsets revealed that the majority of colony-forming cells were present in the CD97NEG (7142±5057 CFU-GM) population compared to CD97HI (178±170CFU-GM) and CD97INT (3047±2902 CFU-GM per 106 BM cells). Analysis of CAFC frequencies of CD97HI, CD97INT and CD97NEG BM cells showed that CAFC-day 35 activity resided in the CD97INT BM population (3.0±1.2 vs. 0.9±0.1 CAFC-day 35 per 105 cells for CD97INT and total BM respectively), whereas no CAFC-day 35 activity was found in CD97HI and CD97NEG BM fractions. In addition, FACS analysis revealed that the majority (82.6%) of c-kitPOSthy-1LOLinNEG/LO cells were present in the CD97INT BM population. To investigate the *in vivo* repopulating ability of the different CD97 BM subsets, lethally irradiated (9.5 Gy) BALB/c recipient mice (n=10 per group) were reconstituted with 105 syngeneic BALB/c total BM or with CD97 sorted BM cells. Repopulating capacity entirely resided in the CD97INT subset (repopulation rate 90% versus 0% in the CD97HI and CD97NEG subset). These data indicate that 1) CD97 is differentially expressed on HPC and HSC and that 2) CD97 expression can be used to separate colony forming cells from repopulating hematopoietic stem cells.

Dendritic cells, vaccination and cellular immunotherapy

0480

CLINICAL BENEFIT ASSOCIATED WITH IDIOTYPIC VACCINATION IN FOLLICULAR LYMPHOMA

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Background. So far, no human tumor vaccine has proved beneficial to any cancer patients. *Aims.* The formal demonstration of such an efficacy is currently widely sought, particularly in the setting of idiotypic vaccination for follicular lymphoma (FL). However, standard randomized trials struggle with the major flaw of experimental arms in which each and every patient ultimately undergoes a different, customized treatment. *Methods.* For this reason, we have instead conducted a phase II study in which first relapse FL patients achieving a second clinical complete response (CR) following monthly CHOP-like chemotherapy (CHT) were to receive up to 10 idiotypic vaccinations over 23-27 months, depending on the duration of the length of time between CHT completion and vaccination start. The duration of this period was based on the time to recovery of normal numbers of circulating CD3+, CD4+, CD8+ and CD19+ lymphocyte subpopulations. From October 2001 to June 2004, thirty-three patients were enrolled. Eight of them ultimately were not vaccinated for a variety of reasons such as failure at producing the vaccine, early relapse or both. In the vaccinated patients, specific humoral responses were assessed by standard ELISA, specific cellular responses by 5 independent methods, and minimal residual disease (MRD) by 3 independent methods. *Results.* Of the 25 patients who were actually vaccinated, 20 have specifically responded to the vaccine with a humoral (13/20) and/or a cellular response (18/20), while five have not. From a clinical standpoint, the median duration of the first clinical CR of the 25 patients vaccinated was 17 months (range: 5-65); their current median follow-up is 21 months after the salvage treatment above. Among them, 19/25 had received at diagnosis a treatment that might be considered comparable in activity to the CHT used at the time of relapse. Moreover, no patient had lower stage, FLIPI score or histological grade at the time of disease recurrence. The median duration of the second clinical CR of the 20 responders has not been reached, but exceeds 29+ months (range: 16+ - 47+). Only 1 vaccinated patient has relapsed at 10 months after the last vaccination and 4 months after the previously documented idiotype-specific and vaccine-induced immune response was no longer detectable. In addition, this relapsed patient had a tumor that expressed an altered idiotype. As frequently seen in FL patients treated for relapse, MRD did not specifically correlate with clinical outcome. Remarkably though, no patient who made a vaccine-induced, sustained and specific immune response relapsed during the 26-30 months of active vaccination (0/20). All responding patients with a sufficient follow-up experienced a second clinical CR longer than both their corresponding first clinical CR (17/17; $p<0.0001$ by both standard and adjusted-for-matching log rank test) and the median duration of a CHOP-induced second clinical CR, generally estimated in 13 months (20/20). By contrast, all 5 patients in whom the vaccine completely failed to elicit an immune response had a second clinical CR shorter than the first clinical CR, and relapsed within 8-13 months after having achieved second remission. *Conclusions.* Taken together, these results provide the first evidence ever of clinical benefit associated with the use of a human cancer vaccine.

0481

RHAMM/CD168-R3 PEPTIDE VACCINATION OF HLA-A2+ PATIENTS WITH ACUTE MYELOID LEUKEMIA, MYELODYSPLASTIC SYNDROME AND MULTIPLE MYELOMA ELICITS IMMUNOLOGICAL AND HEMATOLOGICAL RESPONSES

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Background. The receptor for hyaluronic acid mediated motility (RHAMM/CD168) RHAMM/CD168 is expressed in more than 80% of patients with acute myeloid leukemia (AML) or multiple myeloma (MM). Recently, we characterized RHAMM/CD168 as a leukemia-associated antigen (LAA) eliciting both humoral and cellular immune responses in patients with hematological malignancies. Furthermore, we defined the RHAMM/CD168-derived peptide R3 (ILSLELMKL) as a CD8+ T cell epitope. R3-primed CD8+ T lymphocytes were able to lyse autologous RHAMM/CD168+ AML blasts in a MHC class I-restricted and epitope-specific manner. Aims. We therefore initiated a phase I/II R3 peptide vaccination to induce immunological and hematological responses for patients with AML, MDS or MM overexpressing RHAMM/CD168 to induce immunological and clinical responses. Methods. Patients were included with positive RHAMM/CD168 expression but with a limited tumour load. At a biweekly interval, 300 mcg RHAMM R3 peptide emulsified with the incomplete Freund's adjuvant (day 3) and GM-CSF (100 mcg, days 1-5) was administrated four times subcutaneously. The primary aim of the study is safety and feasibility of this peptide vaccination, secondary aims the evaluation of a specific T cell immune response to RHAMM/CD168 R3 peptide and the assessment of the influence of the R3 peptide vaccination on the remission status. Since January 2005, twelve patients were enrolled in this study. Results. The first ten patients (2 AML, 4 MDS, 4 MM) have completed the course of four vaccinations and four patients have been evaluated. The only side effects observed under R3-peptide vaccination were erythema and induration of the skin at the site of injection (CTC I°). In 7/10 patients, we found in the peripheral blood a significant increase of specific CD8+ T cells (from 0.01% to 0.8%) recognizing the R3 peptide in ELISPOT analysis and seven-color flow cytometry including tetramer staining, two patients showed already initially a high number of HLA-A2/R3 tetramer+WT1tetramer-CCR7-CD27+CD28+CD45RA+ effector T cells and main-tained this level of T cell response. Clinical responses have been assessed by the examination of peripheral blood and bone-marrow samples before and after vaccination. Patients showed a reduction of the tumor-specific expressed antigen RHAMM/CD168 in real-time RT-PCR analysis after vaccination. 3/7 patients with myeloid disorders (1 AML, 2 MDS/RAEB1) showed a reduction of CD33+ cells in FACS analysis of the bone-marrow after four vaccinations from 10 and 7% to 1-2 and <1%, respectively. Two patients with MM showed a reduction of plasma cells in bone-marrow and a stable quantity of light chains in peripheral blood, one patient with AML showed a progressive disease. Conclusion. 70% of immunological and 40% of hematological responses were observed. RHAMM/CD168 is therefore a promising target antigen for immunotherapies in patients with hematological malignancies.

0482

TRIGGERING OF P38 MAPK BY CONDITIONAL MKK6 INDUCTION IS SUFFICIENT FOR DENDRITIC CELL MATURATION, AN EFFECT FURTHER ENHANCED BY INHIBITION OF NUCLEAR RELB

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Activation of Langerhans cells (LCs) by diverse signals involves p38 MAPK phosphorylation. Whether p38 is sufficient to trigger LC activation remains unknown. We show that conditional induction of a dominant active form of MAPK kinase 6 (d.a.MKK6), a direct upstream kinase of p38, in LCs is sufficient to induce the upregulation of co-stimulatory molecules and to enhance their T cell stimulatory capacity. These immediate effects showed no or only a minor requirement for classical NF-κB signaling. Concomitant with LC activation, d.a.MKK6 strongly induced the alternative NF-κB member RelB, whose nuclear localization marks mature DCs. Specific inhibition of nuclear RelB during MKK6-induced LC activation further enhanced their maturation state, thus suggesting a novel LC intrinsic control mechanism regulated by RelB.

0483

THE IMMUNE RESPONSE TO BCR-ABL PEPTIDE IMMUNISATION IS VARIABLE AND TRANSIENT IN CHRONIC MYELOID LEUKAEMIA: RESULTS FROM THE EPIC STUDY

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Chronic myeloid leukaemia (CML) is characterised by the BCR-ABL oncoprotein. The peptide sequences spanning the junctional region are completely leukaemia-specific. Vaccination with these peptides could therefore elicit/augment an immune response directed to CML cells. Entry requirements to our ongoing Evaluation of Peptide Immunisation in CML (EPIC) study were all of the following: first chronic phase of CML, expression of the e14a2 (b3a2) BCR-ABL transcript, and prior treatment with imatinib at a stable dose of at least 400 mg daily for at least 8 months. Each patient received intradermally a cocktail of 3 BCR-ABL peptides: (1) a 9-mer spanning the e14a2 region, (2) this same 9-mer linked to a PADRE (a 15-mer non-natural peptide shown to activate CD4+ T cells, to which all patients are immunologically naive), and (3) a 13-mer consensus e14a2 junctional peptide linked to PADRE. These peptides were administered at either 100 (5 patients), 300 (5 patients), 600 (5 patients), or 1000 μg (3 patients) with sargramostim on 6 occasions over 2 months. Immune responses to the vaccine were monitored by IFN-γ and IL-5 ELISPOT assays on peripheral blood mononuclear cells. Currently, 18 patients are evaluable at 6 months of follow-up. At entry, no patient showed a detectable immune response to the PADRE peptide, but all 18 patients had detectable T cell responses within 3 months of commencing vaccination. These typically persisted at 5 and 6 months (i.e. 3 and 4 months after completion) of vaccination. These anti-PADRE responses were carried out by CD4+ T cells as demonstrated by flow cytometry analysis of IFN-γ-producing cells, and indicated that the vaccination protocol was capable of stimulating T cells in all 18 patients. Immune responses to BCR-ABL junctional peptides were monitored using the 9-mer sequence used in the vaccine and an 18-mer spanning the whole junctional region. In all but one case, there was no evidence of T cell responses to these peptides pre-vaccination. Upon vaccination, IFN-γ-producing cells to the 9-mer peptides were detected in 11/18 patients, and these cells were demonstrated to be CD8+ T cells by flow cytometry analysis. Moreover, CD4+ T cells specific for the 18-mer junctional peptide were detected in 14/18 patients. Interestingly, immunophenotyping indicated that these BCR-ABL-specific T cells were of a memory phenotype (CD45RO+). However, the anti-BCR-ABL responses were typically transient, disappearing by 5 months (i.e. 3 months after completing vaccination) in all but one case, in sharp contrast to the responses to PADRE. A good correlation was observed between the presence of BCR-ABL-specific T cells and a decrease in the level of BCR-ABL transcripts. These data demonstrate that peptide vaccination can elicit anti-BCR-ABL peptide responses in CD8+ and CD4+ T cells, but these are less frequent and less durable than those to the novel antigen PADRE. This suggests that BCR-ABL is either a weak antigen or that patients may be tolerised to it. Further functional characterisation (e.g. granzyme B production) of these BCR-ABL specific T cells is currently ongoing.

0484

MULTIPLE MYELOMA REACTIVE CYTOTOXIC T CELLS RECOGNIZE A NEW ACTIVATION INDUCED MINOR HISTOCOMPATIBILITY ANTIGEN ENCODED BY ATP DEPENDENT INTERFERON RESPONSIVE GENE

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Minor Histocompatibility antigens (mHag) play an important role in both graft versus tumor effects and graft versus host disease (GVHD) after allogeneic stem cell transplantation (SCT). From a patient with multiple myeloma after allogeneic SCT entering complete remission after donor lymphocyte infusion (DLI) coinciding with mild GVHD, several T cell reactivities were isolated at the time of the clinical response. The most dominant T cell reactivity showed HLA-A2 restriction and strongly recognized tumor cells and activated B and T cells (PHA-blasts), whereas resting T cells were only moderately recognized. From mHag positive EBV-LCL peptides were isolated, tested for reactivity and subjected to sequence analysis. One candidate peptide reconstituted CTL reactivity. This peptide was identical to an alternatively translated protein sequence derived from the human ADIR gene. A SNP in this gene resulting in an aminoacid change in the candidate peptide was shown to be present in patient cells. Synthetic peptides of both patient and donor SNP variants were synthesized and specific recognition of the identified patient derived peptide was demonstrated. Transfection experiments with plasmids containing patient or donor ADIR gene constructs confirmed involvement of this SNP in T cell recognition. A population study revealed 100% correlation of the presence of the relevant SNP with recognition of PHA-blasts in 51Cr release assays. The SNP was present in 43 out of 76 individuals tested. We designated the epitope as LB-ADIR-1F. Tetramer analysis of patient samples that were taken after DLI showed up to 2.6% LB-ADIR-1F specific T cells coinciding with conversion to remission. It was previously shown that IFNα could upregulate ADIR gene expression, and the patient was treated with IFNα during DLI. Therefore, SNP positive MNC were cultured with IFNα prior to addition of the LB-ADIR-1F CTL. IFNα increased both susceptibility to lysis and stimulatory capacity of pretreated MNC. Quantitative PCR showed increased ADIR mRNA levels in IFNα stimulated cells thus supporting the role of IFNα in ADIR gene expression. Recognition of SNP positive mesenchymal stem cells as a representative of non hematopoietic cells was low and growth arrest further decreased recognition. Analysis of the immunological response in this patient also revealed T cell reactivities directed to the mHag LB-ECGF-1H and HA1. The sum of the percentages of these circulating mHag specific T cells at the time of the clinical response was 4.3%, approaching the total number of activated circulating CD8+ T cells in the patient as measured by HLA-DR expression suggesting that these 3 reactivities were responsible for the clinical course. Whereas expression of HA1 and ECGF is relatively restricted to hematopoietic cells, ADIR gene expression is more broad. LB-ADIR-1F specific T cells were shown to be highly cytotoxic for multiple myeloma cells and other hematological malignancies. Since only mild acute GVHD was observed which rapidly disappeared after discontinuation of the IFNα treatment and administration of corticosteroids, we hypothesize that the activation status of GVHD target tissues determines the clinical outcome of treatment with LB-ADIR-1F specific T cells in adoptive immunotherapy.

Vascular biology and granulocytes

0485

INHIBITION OF HIF1-A BY A POTENT RNA ANTAGONIST IS ASSOCIATED WITH MULTIPLE MECHANISMS OF ANTI-TUMOUR ACTIVITY

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Substantial evidence has accumulated to demonstrate that over-expression of Hif-1α is associated with tumour angiogenesis, tumour progression and poor prognosis in a broad range of cancers. It thus represents a potential point of intervention for targeted therapeutics for this group of cancers. The unique properties of Locked Nucleic Acid (LNA) chemistry have been used to generate a single stranded RNA antagonist to the Hif-1α mRNA (SPC2968), which exhibits increased stability, improved resistance to nucleases and much higher binding affinity to the target, than other second and third generation oligonucleotides. We demonstrate that SPC2968 potently inhibits Hif-1α expression *in vitro* (IC50 < 1nM) and that this is correlated with parallel inhibition of Hif-1α regulated genes *in vitro* under hypoxic and normoxic conditions. Hif-1α knock down in cancer cell lines was also correlated with increased induction of apoptosis and cell death. Following administration of a single dose of SPC2968 to wild type mice, liver Hif-1α mRNA levels were substantially reduced for periods up to 5 days. Hif-1α inhibition also correlated with reduced expression of genes regulated by Hif-1α, namely MMP2 and VEGF. *Ex vivo* assays of endothelial tube formation and aorta ring outgrowth demonstrated that SPC2968 administration was also associated with impaired ability of endothelial cells to form capillaries and sprout. Potent anti-tumour effects of the drug were also observed in murine xenograft models, both when tumour cells were transfected with SPC2968 prior to implantation and when pre-treated tumours were subsequently treated in the host during the study, suggesting that both initial and later phases of tumour growth can be impeded by Hif-1α down-regulation. The bio-distribution of SPC2968 following intravenous administration, as assessed by whole body autoradiography using tritium-labelled SPC2968, showed extensive tissue distribution with the LNA oligo still detectable in the kidney 21 days after the injection. Fluorescent-labelled SPC2968 distribution and cellular localisation were additionally investigated in several organs including skin, tumor, liver, kidney and bone marrow. All cell lineages tested were found to be positive for the label. Correlation between uptake of SPC2968 and Hif-1α expression was also addressed by HPLC and QPCR analysis in different tissues. Overall we provide data supporting the therapeutic use of a single-stranded, LNA-based RNA antagonist to target Hif-1α, and conclude that this important transcription factor may act at several points in tumour development.

0486

T-CELL RECEPTOR VB REPERTOIRE ANALYSIS IN CHRONIC IDIOPATHIC NEUTROPENIA: EVIDENCE FOR PRESENCE OF PROMINENT T-CELL CLONES WITH PATHOGENETIC SIGNIFICANCE

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Background. Chronic idiopathic neutropenia (CIN) is an acquired underproduction neutropenia syndrome characterized by hypoplastic and left-shifted granulocytic series in the bone marrow (BM). We have previously shown that CIN patients display increased number of activated T-lymphocytes with myelosuppressive properties in the BM and peripheral blood (PB) that induce Fas-mediated apoptotic death of the myeloid progenitor cells by producing interferon-γ and Fas-ligand. Epidemiological data have also shown an association of CIN with HLA-DRB1*1302 genetic background. *Aims.* To characterise T-cell receptor (TCR) Vb repertoire of patients with CIN seeking for dominant T-cell expansions with possible pathogenetic significance. *Methods.* Fifty-nine patients with CIN were studied. All patients had neutrophil counts below 1800/μL (mean 1411±380/microliter, range 100-1799 neutrophils/microliter) and were satisfying the previously reported diagnostic criteria for the disease. PB samples from the patients were subjected to flow-cytometric analysis for the quantification of the TCR Vb repertoire of the CD3+ cells (IOTest β Mark kit, Beckman-Coulter). Vb family expansions were defined as above of 2SD (standard deviation) from the means in 85 healthy controls. Blood DNA samples were also subjected to multiplex PCR using the BIOMED2 protocol that covers all Vb TCR gene rearrangements. *Results.* Forty-four of the patients, i.e. a proportion of 74.6% displayed expanded Vb subsets with Vb16 _and Vb12 representing the most frequent expanded clones. Specifically, the patients as a group displayed statistically significant increased proportion of Vb16 and Vb12 expressing T-cells (2.17%±1.40% and 2.62%±2.83%, respectively) compared to controls (0.90%±0.29% and 1.66%±0.54%, respectively) (p<0.001 and p<0.01, respectively). These TCR-Vb over-representations were associated with a parallel under-representation of Vb5.3 (p<0.05), Vb7.1 (p<0.001), Vb9 (p<0.001), Vb17 (p<0.001), Vb18 (p<0.001), Vb5.1 (p<0.001), Vb13.1 (p<0.01), Vb13.6 (p<0.02), Vb5.2 (p<0.01), Vb2 (p<0.001), Vb14 (p<0.02), Vb11 (p<0.05), Vb22 (p<0.001), Vb4 (p<0.001) expressing T-cells compared to controls. None of the patients displayed monoclonal TCR-Vb expansion in multiplex PCR analysis. *Summary-Conclusions. in vivo* dominant T-cell responses are identified in the majority of patients with CIN. These data substantiate further the hypothesis for the immune nature of CIN providing therefore novel insights in the pathophysiology of the disease with possible therapeutic implications, i.e. immunosuppressive therapy in severely neutropenic patients. The cloning and sequencing of the complementarity-determing region 3 (CDR3) of the expanded Vb subsets is under investigation to identify, if any, specific antigen-driven T-cell responses in CIN patients.

0487

CONCURRENT MUTATIONS IN NEUTROPHIL ELASTASE AND GRANULOCYTE COLONY-SIMULATING FACTOR RECEPTOR GENES IN A CASE OF SEVERE CONGENITAL NEUTROPENIA

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Background. Severe congenital neutropenia (SCN) is a heterogeneous disorder characterized by extremely low levels of circulating neutrophils, and a propensity for myelodysplastic syndrome and acute myeloblastic leukemia. Germline mutations in the ELA2 gene, encoding neutrophil elastase, are the cause of the disease in 65-80% of the cases. In contrast, mutations in the CSF3R gene, encoding granulocyte colony-stimulating factor receptor (G-CSF-R), are found in approximately 20% of SCN patients and are almost universally acquired. They typically lead to truncation of the intracellular domain of the receptor and result in extended signaling, particularly of STAT5, that may play a role in the predisposition of SCN patients to leukemia. However, we have previously described an SCN patient with a constitutive mutation in the G-CSF-R extracellular domain that results in hyporesponsiveness to ligand and suppressed STAT5 signaling. *Aims.* To further our understanding of SCN etiology through a re-examination of this patient with respect to the status of the ELA2 and CSF3R genes. *Methods.* Genomic DNA and hematopoietic cell-derived cDNA were analysed for the presence of mutations in the ELA2 and CSF3R genes. Compound G-CSF-R mutants were then examined for their ability to activate STAT5. *Results.* A novel germline ELA2 mutation was identified in this patient, causing a frameshift after P205 and a premature stop. In addition, two independent truncating mutations within the G-CSF-R intracellular domain, R710X and Q718X, were detected at different times in this patient. *in vitro* studies demonstrated that such intracellular truncations could partially restore the STAT5 response in the context of the extracellular P206H mutation. *Summary/Conclusions.* These data add to our understanding of the etiology of SCN adding to the evidence that ELA2 mutations are a likely primary cause. These may be exacerbated by CSF3R mutations, particularly those in the extracellular domain that affect responsiveness to G-CSF, with the neutropenic environment conducive to the subsequent expansion of cells expressing truncating G-CSF-R mutations. In addition, these results further attest to the importance of STAT5 in mediating responses to G-CSF.

0488

NEOPLASTIC CIRCULATING ENDOTHELIAL CELLS IN HEMATOLOGIC MALIGNANCIES

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Background. Several studies have shown that bone marrow-derived endothelial cells (EC) may contribute to tumor angiogenesis and that in the peripheral blood of cancer patients there is an increased amount of circulating ECs (CECs) that may participate to new vessel formation. Recent data also showed that microvascular ECs in B-cell lymphomas are in part tumor-related reflecting a novel aspect of tumor angiogenesis. All together these observations suggest that tumors can elicit the sprouting of new vessels from existing capillaries through the secretion of angiogenic factors and that, in some cases, cancer cells can also mimic the activities of ECs by participating in the formation of vascular-like networks. *Aims.* To clarify if, in different hematologic malignancies with known cytogenetic aberrations, CECs are tumor-derived. *Methods.* We studied 21 patients with different hematologic malignancies (6 MM, 2 CML, 5 AML, 1 ALL and 7 CLL). To isolated CECs, we used a dual step immunomagnetic sorting by means of CD45 and CD146 antibodies. By using immunomagnetic sorting in combination with CD45, we first eliminated all hematopoietic cells, which are CD45 positive, without affecting the EC component, which is characteristically CD45 negative. We then sorted CECs by means of CD146, an antigen expressed almost exclusively on ECs and absent on hematopoietic cells. To confirm the EC commitment, we then performed additional phenotypic studies with antibodies recognizing endothelial and neoplastic cells. FISH analysis was finally performed on sorted CECs with different commercially available probes in dual colour experiments. *Results.* In all experiments more than 95% of immunomagnetically sorted cells were of EC origin as demonstrated by phenotypic analyses. After immunomagnetic selection less than 0.5% of cells were CD45+ while CD14 was expressed in 0.1% of all immunomagnetically sorted CECs. More than 95% of immunomagnetically sorted CECs expressed VEGFR2, vWf, CD144 and UEA-1 lectin. Very few immunomagnetically sorted CECs expressed antigens expressed on neoplastic cells (CD138, CD38, CD33, CD19, CD5). FISH analysis showed that a significant proportion of CECs was tumor-derived because they harbored the same genetic lesion as observed in neoplastic cells. The fraction of CECs showing the cytogenetic aberration averaged 20% (range, 11-34%, 200 cells observed in each case). The majority (>85% of CECs presented features of EPCs because they expressed CD133, a marker gradually lost during EC differentiation and absent in mature ECs. Overall, 98.0% of CECs with genetic lesions were CD133 positive. *Conclusions.* These findings suggest that in many hematologic malignancies CECs are in part tumor related and with EPC features. These CECs may contribute to tumor neovasculogenesis and possibly to the spreading and progression of the disease. It is possible to speculate that neoplastic CECs may have arisen from a common hemangioblast precursor that can give rise to both neoplastic cells and ECs or alternatively through a process of dedifferentiation of a already committed cell into a cell with EPC characteristics followed by a redifferentiation into a terminally differentiated EC. Disguised plasma cells may then mimic functional CECs and contribute to tumor neovasculogenesis.

0489

EVIDENCE FOR DISTINCT PHENOTYPIC STAGES DURING ENDOTHELIAL PROGENITOR DIFFERENTIATION

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Background. Despite the importance of endothelial progenitor cells (EPC) in tumor angiogenesis, little is know about the mechanisms that regulate their differentiation towards mature endothelium (EC). *Aims.* In the present project, we hypothesized the process of EPC>>EC differentiation might involve distinct phenotypic/functional phases, which may be characterized by the expression of particular genes/gene products and thus may be suitable for therapeutic targetting. *Methods.* To define whether this is the case, we conducted cDNA/ oligonucleotide microarrays (Superarray and Affymetrix) analysis of human umbilical cord blood CD34+ cells, cultured in EC differentiation medium, at different timepoints after the start of culture. *Results.* The initial characterization (day 0) of freshly isolated CD34+KDR+CD117+ cord blood cells revealed a gene expression pattern, which included stress related genes (tnf, ifnα1, tgfβ1), those coding for matrix-specific receptors (Integrins α 1 and 5) and some involved in particular signalling pathways (Raf1, IkBα). As the cells respond to the growth factors in the culture medium, the first stage of EC differentiation (day 5) revealed an increase in the expression of specific integrins, receptors (Fgfr2, Flt1, Tek), an up-regulation of cell cycle- (Cdkn1b, Rad53, Ccnd1), and apoptosis-related genes (Bcl2l1, Tnfrsf1a, Casp8). In contrast, genes indicative of mature EC function (Il-8, thrombospondin 2, Timp1) were expressed solely at the end of the differentiation assay (days 23-26). *Summary/ Conclusions.* We suggest the following stages regulate endothelial differentiation from EPC: 1) adherence to a particular matrix; 2) response to specific growth factors, promoting proliferation and survival; 3) maturation and acquisition of endothelial functions. These results reveal new putative targets with therapeutic potential, and suggest that strategies aimed at blocking EPC recruitment and differentiation (to halt tumor angiogenesis), should be designed specifically against defined target genes. We are now performing RNAi studies to test the function of specific genes (integrins, chemokines) during endothelial differentiation.

Multiple Myeloma - Clinical

0490

A PROSPECTIVE RANDOMIZED CONTROLLED TRIAL OF ORAL MELPHALAN, PREDNISONE, THALIDOMIDE VERSUS ORAL MELPHALAN, PREDNISONE IN ELDERLY NEWLY DIAGNOSED MYELOMA PATIENTS

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Background. For patients older than 65 years of age oral melphalan and prednisone (MP) has remained the treatment of choice since 1960. So far no major improvement in outcome from the original combination MP has been achieved in these elderly patients and new treatments are urgently needed. In this multicentre randomised trial we compared oral melphalan and prednisone plus thalidomide (MPT) with MP alone in 60 to 85 years old patients. *Aims.* The primary objective was to compare the clinical response rates and the event-free survival in the two treatment groups. Secondary end points included overall survival, prognostic factors, time to the first evidence of response and incidence of any grade 3 or higher adverse events. *Methods.* The trial was conducted at 54 centres in Italy. Patients with newly diagnosed multiple myeloma were randomly assigned to receive oral MP (N=129) for six four-week cycles plus thalidomide 100 mg per day continuously until any sign of relapse or progressive disease (Pharmion LTD, Windsor, UK) or MP alone (N=126). The dose of thalidomide was reduced 50% on the occurrence of any non-haematological grade 2 toxicity and it was discontinued for any non-haematological grade 3 toxicity. No anticoagulation prophylaxis was administered until December 2003 when the protocol was amended and enoxaparin at 40 mg per day was delivered subcutaneously during the first four cycles of therapy. *Results.* Patients treated in MPT arm experienced higher response rates and a longer event-free survival than patients who were not. In intention-to-treat analysis, the complete and partial response rates were 76.0% for MPT and 47.6% for MP alone (absolute difference +28.3%, 95% CI 16.5 to 39.1), and the near complete and complete response rates were 27.9% and 7.2%, respectively. The two-year event-free survival rate was 54% in patients receiving MPT and 27% in patients receiving MP. The hazard ratio (HR) for MPT was 0.51 (95% CI 0.35 to 0.75), $p<0.001$. This is a 49% decrease in the risk of events in the MPT group. The three-year survival rate was 80% in patients taking MPT and 64% in patients taking MP, the HR for MPT was 0.68 (95% CI 0.38 to 1.22), $p=0.19$. Grade 3-4 adverse events were 48% in MPT patients and 25% in MP patients ($p<0.001$). In the MPT group, the most frequent grade 3-4 adverse events were haematological, thromboembolism, infections and peripheral neuropathy. The introduction of enoxaparin prophylaxis significantly reduced the incidence of thromboembolism from 20% to 3% ($p=0.005$). *Conclusion.* Oral MPT is superior to MP as first-line treatment for elderly patients with multiple myeloma. Anticoagulant prophylaxis reduced the frequency of thrombosis. Longer follow-up is needed to assess the effect on overall survival.

0491

ORAL REVLIMID PLUS MELPHALAN AND PREDNISONE FOR NEWLY DIAGNOSED MULTIPLE MYELOMA: A PHASE I-II STUDY

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Background. Lenalidomide (Revlimid®) is a novel, orally active immunomodulatory drug effective for the treatment of relapsed and refractory myeloma. Lenalidomide has shown additive effects with melphalan and corticosteroids. No data are available on the clinical use of Lenalidomide in combination with the oral melphalan and prednisone (MP). *Aims.* In this multicenter phase I-II study, we evaluate the safety and efficacy of different doses of Revlimid® in combination with melphalan and prednisone (R-MP). *Methods.* Between December 2004 and November 2005, 54 newly diagnosed symptomatic MM patients (pts) older than 65 years were enrolled. Pts were treated with 9 courses of Revlimid_ (5-10 mg/day for 21 days every 4-6 weeks) plus MP (melphalan 0.18-0.25 mg/kg/day and prednisone 2 mg/kg/day for 4 days every 4-6 weeks). Four different dose levels were tested: 1. melphalan 0.18 mg/kg + Revlimid® 5 mg/day; 2. melphalan 0.25 mg/kg + Revlimid® 5 mg/day; 3. melphalan 0.18 mg/kg + Revlimid® 10 mg/day; 4. melphalan 0.25 mg/kg + Revlimid® 10 mg/day. Each cohort included 6 pts, with additional 15 pts enrolled at dose level 3 and 4. Dose limiting toxicity (DLT) was defined as: any grade > 3 non-hematologic toxicity; grade 4 neutropenia lasting >7 days; any other grade 4 hematologic toxicity and any treatment delay due to toxicity that occurred during the first cycle. All pts received ciprofloxacin and aspirin as prophylaxis. *Results.* 50 pts (median age 71, range 57-77) were evaluated after at least one R-MP course. No DLTs were observed in the first 2 dose levels. In level 3 one pt experienced DLT (grade 4 neutropenia lasting> 7 days). In level 4 three pts showed DLTs (1 pt experienced neutropenic fever and grade 3 cutaneous toxicity, 1 pt had pulmonary embolism, 2 pts had a delay in the start of cycle 2 due to hematologic toxicities). After 1 cycle of R-MP, no one was in complete remission (according to the EBMT/IBMTR criteria), 16% of pts showed myeloma protein reduction of 75-99%, 35% myeloma protein reduction of 50-74%, and 49% reduction <50%, no disease progressions were observed. After 3 cycles of R-MP, complete remission was observed in 10% of pts, myeloma protein reduction of 75-99% was detected in 30%, response of 50-74% in 30% and response <50% in 30%, no disease progressions were observed. Major grade 3 or 4 adverse events consisted of hematological toxicities: neutropenia (58%), thrombocytopenia (21%) and anemia (13%). Major grade 3 or 4 non-hematological toxicities recorded were cutaneous eruption (11%), infection (5%) and febrile neutropenia (3%). Neuropathy was not observed and only one case of tromboembolic events (pulmonary thromboembolism) was recorded. *Conclusions.* R-MP represents a feasible and promising approach for newly diagnosed pts who are not candidates for transplant. It was well tolerated with a manageable toxicity and showed a significant response rate. An update of these data will be presented.

0492

HAEMATOLOGICAL PROFILES WITH BORTEZOMIB OR HIGH-DOSE DEXAMETHASONE TREATMENT IN RELAPSED MULTIPLE MYELOMA: PHASE 3 APEX TRIAL

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Background. Bortezomib (VELCADE®) is a novel proteasome inhibitor that has demonstrated safety and efficacy for patients with relapsed and/or refractory multiple myeloma in phase 2 and 3 trials. Bortezomib was associated with thrombocytopenia and neutropenia in SUMMIT (NEJM. 2003;348:2609) and CREST (BJH. 2004;127:165) and both were transient and cyclical. *Aims.* This analysis characterised the haematological profiles of patients treated with bortezomib or high-dose dexamethasone in APEX, the largest phase 3 trial in patients with relapsed multiple myeloma following 1-3 prior therapies (NEJM. 2005;352:2487). *Methods.* 669 patients with relapsed multiple myeloma were randomised to bortezomib 1.3 mg/m^2, d 1, 4, 8, 11 q3wk for 8 cycles, then 3 cycles on d 1, 8, 15, 22 q5wk, or dexamethasone 40 mg, d 1-4, 9-12, 17-20 q5wk for 4 cycles, then 5 cycles on d 1-4 q28d. Data on adverse events, laboratory values, and transfusion experience were collected at baseline and regularly through therapy. *Results.* Anaemia, neutropenia, and thrombocytopenia reported as adverse events are shown (Table). The incidence of anaemia was similar in both treatment arms. 33% of patients on bortezomib and 20% of those on dexamethasone received blood transfusions for anaemia. Patients on bortezomib experienced a steady increase in haemoglobin over time and the requirement for blood transfusions decreased over time to 0% after cycle 4. Bortezomib-associated neutropenia was also transient and cyclical, and febrile neutrope-

nia was rare. G-CSF or GM-CSF was used at a low rate to manage neutropenia. Thrombocytopenia was cyclical, with recovery towards baseline during the rest period of each cycle. Overall, 15% of patients on bortezomib and 1% of those on dexamethasone received platelet transfusions for thrombocytopenia. Preclinical study of the effect of bortezomib on megakaryocytes indicates a shorter recovery time than with cytotoxic marrow injury, an absence of a lethal cytotoxic effect, and no cumulative or persistent thrombocytopenia. The number of patients requiring platelet and blood transfusions peaked within the first 2 cycles in both treatment arms. Although the number of platelet transfusions was higher with bortezomib, the number of significant bleeding events (including any grade 3/4, any with an intensity reported as serious, and cerebral haemorrhage regardless of intensity and seriousness) was similar in the 2 arms. No difference was observed in response rate or duration of response in patients who received platelet transfusions compared with patients who did not need platelet transfusion. Median duration of therapy for platelet-transfused patients was 3.8 and 3.4 mo in the bortezomib and dexamethasone arms, respectively. *Conclusions.* Haematological adverse events with bortezomib are predictable and manageable. The kinetics and mechanism appear different from those observed with standard cytotoxic therapy. Neutropenia was transient and rapidly recovered to baseline during the rest period of each bortezomib treatment cycle, with few patients requiring growth factor support. Thrombocytopenia was also transient and reversible. When clinically indicated, platelet transfusion rather than dose reduction or treatment interruption may be warranted to maximise the benefit of bortezomib therapy.

Table 1.

Event	*Bortezomib (n=331)*	*Dexamethasone (n=332)*
Anaemia, n (%)		
All grades	87 (23)	74 (22)
G3/4	33 (10)	35 (11)
Neutropenia, n (%)		
All grades	62 (19)	5 (2)
G3/4	48 (14)	4 (1)
Thrombocytopenia, n (%)		
All grades	115 (35)	36 (11)
G3/4	97 (30)	22 (6)

0493

LENALIDOMIDE (REVLIMID) IN COMBINATION WITH DEXAMETHASONE IS MORE EFFECTIVE THAN DEX ALONE IN PATIENTS WITH RELAPSED OR REFRACTORY MULTIPLE MYELOMA WHO HAVE RECEIVED PRIOR THALIDOMIDE THERAPY

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Background. Lenalidomide is a novel, oral, immunomodulatory drug that is effective against multiple myeloma. In the prospective, randomized, placebo-controlled phase III trials MM09 and MM010, lenalidomide with dexamethasone induced a significantly higher response rate and complete remission rate, as well as longer time-to-progression (TTP) in comparison with dexamethasone alone. *Aim.* The current prospective subgroup analysis was designed to assess whether prior treatment with thalidomide would affect the sensitivity of multiple myeloma to subsequent lenalidomide treatment. *Methods.* A total of 692 patients who had received 1 to 3 prior treatments and were not refractory to dexamethasone were randomized to receive either oral lenalidomide (25 mg daily for 3 weeks every 4 weeks) plus dexamethasone (40 mg on days 1-4, 9-12, 17-20 every 4 weeks for 4 cycles, then 40 mg on days 1-4 every subsequent cycle) (Len/Dex) or placebo plus dexamethasone (Dex alone). The European Blood and Marrow Transplantation criteria were used to evaluate TTP, complete response (CR), and partial response (PR). Randomization was stratified at entry by number of prior therapies (1 versus > 1). *Results.* Among the 269 patients who had received prior thalidomide treatment, those receiving Len/Dex (N=124) had a longer median TTP (36.9 vs. 19.7 wks, $p<0.001$) and a higher response rate (CR + PR; 53.2% vs. 15.2%,$p< 0.001$) versus those receiving Dex alone (N= 145). Complete response rates were also higher with Len/Dex than with dexamethasone in patients who had received prior thalidomide (8.1% vs. 1.4%, $p<0.05$). After a median follow-up of 11 months for all patients, there was a trend for Len/Dex to provide improved overall survival (hazard ratio 1.53, $p=0.0713$) versus Dex alone. *Conclusions.* Lenalidomide in combination with dexamethasone is more effective than dexamethasone alone in patients with relapsed or refractory multiple myeloma who have received prior thalidomide therapy.

0494

LENALIDOMIDE (REVLIMID) COMBINATION WITH DEXAMETHASONE IS MORE EFFECTIVE THAN DEX ALONE IN PATIENTS WITH RELAPSED OR REFRACTORY MULTIPLE MYELOMA AND INDEPENDENT OF NUMBER OF PREVIOUS TREATMENTS

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Background. Lenalidomide is a novel, oral, immunomodulatory drug that is effective against relapsed or refractory multiple myeloma. In the prospective, randomized, placebo-controlled phase III trial MM-010, lenalidomide with dexamethasone induced a significantly higher response rate and complete remission rate, as well as longer Time-to-Progression (TTP) in comparison with dexamethasone alone. The trial included patients who had more than 1 previous unsuccessful regime. *Aim.* To further investigate whether one or more prior therapies influence the TTP between refractory multiple myeloma in patients treated with lenalidomide and dexamethasone or dexamethasone alone. *Methods.* This post hoc analyses included 351 patients who had received 1 to > 3 prior treatments and were not refractory to dexamethasone. The patients were randomized to receive either oral lenalidomide (25 mg daily for 3 weeks every 4 weeks) plus dexamethasone (40 mg on days 1-4, 9-12, 17-20 every 4 weeks for 4 cycles, then 40 mg on days 1-4 every subsequent cycle) (Len/Dex) or placebo plus dexamethasone (Dex alone). Standard criteria were used to evaluate the median TTP. Confidence intervals (based on Kaplan-Meier estimates), hazard ratios (HR; proportional hazards model), and differences between treatment groups (one-tailed log-rank test of survival curve differences between the treatment groups) were calculated. *Results.* Of the 351 patients, 4% had previously received bortezomib, 34% had received thalidomide, 67% had received dexamethasone, and 55% of patients had received stem cell transplantation treatment. A total of 63 patients had 1 prior treatment (n = 30 Len/Dex, n = 33 Dex), 130 patients had 2 prior treatments (n = 65 Len/Dex, n = 65 Dex), and 158 patients had at least 3 prior treatments (n = 81 Len/Dex, n = 77 Dex). Median TTP for patients with 1 prior regimen was not yet reached (NE) (95% CI, 24.1-NE) in Len/Dex patients vs 20.1 weeks (95% CI, 12.9-39.9) in Dex alone patients (HR, 2.8; $p=0.003$). Median TTP for patients with 2 prior regimens was 78.0 weeks (95% CI, 42.4-NE) in Len/Dex patients vs 20.1 weeks (95% CI, 13.3-24.1) in Dex alone patients (HR, 3.7; $p< 0.001$). Median TTP for patients with 3+ prior regimens was 40.9 weeks (95% CI, 32.1-52.4) in Len/Dex patients vs 20.1 weeks (95% CI, 16.1-20.9) in Dex alone patients (HR, 2.5; $p<0.001$). *Conclusion.* Lenalidomide in combination with dexamethasone is more effective than dexamethasone alone in patients with relapsed or refractory multiple myeloma irrespective of the number of prior unsuccessful therapies. TTP after Len/Dex appears to be longer when this combination is used as second line treatment than in later phases of the disease. However, further studies are needed to confirm this observation and to determine whether lenalidomide should be considered sooner in the treatment of multiple myeloma.

Chronic myeloproliferative disorders

0495

EVIDENCE THAT THE JAK2 V617F MUTATION AND MITOTIC RECOMBINATION OCCUR IN A LYMPHO-MYELOID PROGENITOR IN POLYCYTHAEMIA VERA AND IDIOPATHIC MYELOFIBROSIS

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Background. The JAK2 V617F mutation has recently been described as an essential oncogenic event associated with Polycythaemia Vera (PV), Idiopathic Myelofibrosis (IMF) and Essential Thrombocythaemia (ET). This mutation has been detected in all myeloid lineages but has not been yet detected in lymphoid cells. This raises the question whether this molecular event occurs in a true lymphoid/myeloid progenitor cell, as it has already been shown that at least some of these myeloproliferative disorders (MPD) arise from a multipotent stem/progenitor cell. *Aims.* Our aim was to study the presence of the mutation in both myeloid and lymphoid lineages in JAK2 V617F positive MPD. We therefore looked for the mutation first in mature myeloid and lymphoid cells and second in lymphoid/myeloid progenitor cells after CD34+ cell isolation from peripheral blood or bone marrow aspiration. *Methods.* Ten IMF, 12 PV and 6 ET patients harbouring the mutation were enrolled in the study after informed consent. Peripheral blood granulocytes and platelets were purified by standard methods and B, T, NK cells and monocytes were isolated by combined immunomagnetic and flow cytometric procedures. The same techniques were used to sort CD34+ and CD34+CD38- cells from peripheral blood (IMF patients) or from bone marrow mononuclear cells (PV and ET patients). Clonal B/NK/Myeloid differentiation from CD34+CD38- cells and T cell differentiation from CD34+ cells were performed respectively onto a MS5 layer in the presence of SCF, FLT3L, IL2, IL3, IL7, IL15, TPO and in murine Fetal Thymic Organ Cultures (FTOC). Genotyping of mature cell populations, B/NK/Myeloid clones and CD34+ derived T cells were performed by sequencing and/or Taqman® real time allele specific PCR using competitive probes. *Results.* The JAK2 V617F mutation was present in granulocytes and platelets from all patients, and in monocytes from PV and IMF patients. We detected the mutation in B and NK cells from approximately half IMF patients (respectively 4/7 and 5/8 patients), a minority of PV patients (respectively 1/10 and 1/10 patients), and none of the ET patients. Moreover, 2/8 IMF patients had mutated peripheral T cells whereas none of the PV and ET patients did. The JAK2 V617F mutation could be subsequently detected in CD34+ cells and in B/NK/Myeloid and/or NK/Myeloid CD34+CD38- derived clones from all IMF (n=3), PV (n=5) and ET (n=1) patients, with a much higher frequency in clones derived from IMF. Interestingly, a bi-allelic (homozygous) JAK2 V617F mutation was detected in B/NK/Myeloid and/or NK/Myeloid clones from 2 IMF and 3 PV patients, demonstrating the occurrence of the mitotic recombination in a lymphoid/myeloid progenitor cell. Using the FTOC assay, the mutation was also detected in all T cell fractions derived from CD34+ cells from 5 IMF and 2 PV patients. *Conclusions.* These results demonstrate that the mutation and the subsequent mitotic recombination leading to a homozygous subclone occur in a lymphoid/myeloid progenitor cell in JAK2 V617F positive MPD. Thus, the phenotype of these MPD arising from a true lymphoid/myeloid progenitor cell may be related to a downstream selective proliferative advantage of the myeloid lineages.

0496

INDOLENT MYELOFIBROSIS WITH MYELOID METAPLASIA: SPECIFIC CLINICAL FEATURES AND PROGNOSIS

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Background. Patients with myelofibrosis with myeloid metaplasia (MMM) have a heterogeneous prognosis, only partially predicted by published scores and parameters. *Aims.* To identify MMM patients with a very good prognosis, who, therefore, deserve to be excluded from intensive chemotherapy regimens. *Methods.* A prospective national cohort of 871 consecutive patients with MMM was considered. Cluster analysis (EM-algorithm) allowed to classify patients based on their clinical parameters at diagnosis: age, hemoglobin, leukocyte and platelet count, spleen size. Kaplan-Meier survival analysis was applied to the clusters. *Results.* Five clusters were identified: all showed a significantly different clinical phenotype and survival (p<0.0001). Twenty-nine percent (n=250) of the patients were assigned to the cluster with the highest survival, e.g. *indolent MMM*. Their median age was 61 years and 38% were females. Twenty-eight percent of the patients with indolent MMM were absolutely asymptomatic, 19% reported a previous essential thrombocythemia and a few polycythemia vera. All the patients with indolent MMM showed at least one of the following features at diagnosis: hemoglobin values >11 g/dL, platelet count >350Taqman×10^9/L, spleen size <4 cm from costal arc, CD34 count <100/mcl. No patient showed circulating blasts and 90% of the patients showed <2% circulating erythroblasts and <10% circulating immature myeloid cells. A specific rule for selecting patients with indolent MMM includes the presence of a limited splenomegaly (<6 cm from costal arc) associated with: a high platelet count (>600×10^9/L) and/or a normal hemoglobin value and/or an age lower than 65 years. A lower frequency of homozygote JAK2 V617F mutation was detected in patients with indolent-MMM as compared with the rest of the patients (4% vs 31%; p=0.01), irrespectively of previous polycythemia vera. A few patients died of causes directly related to MMM and five-year survival of patients with indolent MMM was 78%. Five-year survival of patients with indolent MMM was higher than overall patients with a low-risk disease, according to the Lille score (p=0.009). Survival did not depend on sex or comorbidity, but depended on age at diagnosis (p<0.0001), five-year survival being >95% in patients aged <40 years, but <75% in patients aged >70 years. Absolute excess mortality, as compared with age-adjusted general population, was 7%, but among patients aged >70 years, absolute excess mortality increased up to 30%. Among patients aged <70 years, females incurred a twice a high excess mortality than males. Percent circulating immature cells was the only clinical parameter that independently predicted survival (p=0.03). Survival was not significantly different in patients followed by a hematology unit as compared with patients followed at an internal medicine unit. *Conclusions.* Cluster analysis on a high number of MMM patients disclosed a population with a very good prognosis. Patients with indolent MMM should not be assigned to front-line intensive therapies.

0497

COEXPRESSION OF JAK2 V617F AND TYPE I CYTOKINE RECEPTORS IS NOT SUFFICIENT FOR CYTOKINE-INDEPENDENT CELL GROWTH

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Background. An activating point mutation in the JH2 domain of Janus kinase 2 (JAK2) was recently described in chronic myeloproliferative disorders (MPD). The majority of patients with polycythemia vera, and substantial numbers of patients with essential thrombocythemia and idiopathic myelofibrosis carry the JAK2 V617F mutation. *Aims.* We set out to find cell lines with histories of MPD expressing the JAK2 V617F mutation, which may be used as suitable tools to analyze basic aspects of the cell biology of these tumors. It has recently been reported that coexpression of type I cytokine receptors with JAK2 V617F proteins leads to cytokine-independence in BA/F3 cells. Our aim was to confirm or refute this correlation in human JAK2 V617F-positive cell lines. *Methods.* Cell lines were tested for the JAK2 V617F mutation applying the PCR-based ARMS assay, confirmed by sequencing, and restriction analysis applying the JAK2 wild-type specific restriction enzyme BsaXI.

Expression and phosphorylation status of JAK2 proteins was checked by immunoprecipitation and Western blot analysis. Cytokine-dependency and influence of JAK kinase inhibitors on cell growth was assayed monitoring 3H-thymidine uptake. Apoptotic cells were detected and quantitated with the annexin-V / propidium iodide method. *Results.* Five / 79 acute myeloid leukemia-derived cell lines tested expressed the JAK2 V617F mutation, 4/5 with histories of MPD. While cell line SET-2 expressed both mutant (mu) and wild-type (wt) JAK2, the remaining positive cell lines carried homo-/hemizygous JAK2 mutations. Microsatellite analysis confirmed losses of heterozygosity (LOH) affecting the JAK2 region on chromosome 9p in the homozygously JAK2mu cell lines HEL, MB-02, MUTZ-8 and UKE-1. Confirming the importance of the mutated JAK2 protein for growth and prevention of apoptosis, JAK2mu cell lines displayed higher sensitivities to JAK2 inhibition than JAK2wt cell lines. It has recently been reported that JAK2 V617F proteins require coexpression of type I cytokine receptors to secure cytokine-independent activation of the JAK2 and STAT5 pathways and to cause cytokine-independent growth of BA/F3 cells. However, 2/5 JAK2mu human AML cell lines described by us were cytokine-dependent, growing after stimulation of type I cytokine receptors: cell line MB-02 responded to erythropoetin and cell line MUTZ-8 responded to G-CSF. Therefore, coexpression of JAK2 V617F and type I cytokine receptors alone is not sufficient for cytokine-independent cell growth. Immunoprecipitation assays showed that JAK2mu cell lines (HEL, SET-2), but not JAK2wt cell lines (SKM-1, SKNO-1), exhibited constitutive phosphorylation of JAK2. Also the cytokine-dependent JAK2mu cell line MUTZ-8 showed constitutive JAK2 phosphorylation. However, short-term stimulation with G-CSF induced phosphorylation of JAK2 in cytokine-starved JAK2mu MUTZ-8 cells, demonstrating that the JAK2 V617F protein was still responsive to G-CSF stimulation. *Summary/Conclusions.* In summary, our results show (i) that coexpression of JAK2 V617F and type I cytokine receptors is not sufficient for cytokine-independent cell growth and (ii) that JAK2 V617F responds to stimulation of type I cytokine receptors.

0498

ASSESSMENT OF MYELOID CLONALITY IN FEMALE PH-NEGATIVE MYELOPROLIFERATIVE DISORDERS BY JAK2V617F MUTATION AND HUMARA ASSAY

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Background. Essential thrombocytemia (ET), polycythemia vera (PV) and agnogenic myeloid metaplasia (AMM) are chronic myeloproliferative disorders (MPDs) arising from clonal hematopoietic stem cells. The detection of JAK2V617F mutation and myeloid clonality by analysis of the human androgen receptor gene (HUMARA), are useful tools to demonstrate clonality in these MPDs. *Aim.* The aim of this study was to compare JAK2V617F mutational status with myeloid clonality determined by HUMARA assay in a series of female patients with Ph-negative MPDs. However, these biomarkers are still not incorporated in the current ET, PV and AMM diagnostic criteria. *Patients and Methods.* One hundred and nine females with Ph-negative MPDs (65 ET, 37 PV and 7 AMM [3 of them being post-thrombocythemic myeloid metaplasia]) from a single institution were studied. Patients fulfilled the diagnostic criteria of PVSG for ET and PV and the Italian criteria for AMM. The median age of ET, PV and AMM patients was 64 (range 25-87), 60 (range 25-84) and 57 (range 53-71) years respectively. At the time of HUMARA assessment, 27/65 ET, 12/37 PV and 2/7 AMM patients were receiving therapy with myelosupressive/platelet lowering agents: hydroxyurea ±ASA (n=30); anagrelide±ASA (n= 10) and busulfan (n=1). Twenty-one patients only received ASA and 47 patients did not receive any specific treatment. HUMARA assay was performed on DNA from purified granulocytes and CD3+ lymphocytes as control cells. The percentage of clonality was calculated after correcting for the degree of lyonization in CD3+ cells. Clonality was established when the corrected allele ratio was < 0.30. Analysis of JAK2V617F was performed by direct sequencing and allele-specific PCR using granulocyte RNA. *Results.* Ninety-two out of 109 (84.4%) females were heterozygous at the HUMARA locus. Forty patients were clonal (18 ET, 16 PV and 6 AMM), 42 patients were polyclonal (31 ET and 11 PV) and ten patients showed allelic skewing. JAK2V617F mutation was identified in 64/109 (58.7%) patients as follows: 31/65 (47.6%) ET patients, 31/37 (83.7%) PV patients and 2/7 (28.5%) AMM patients. When we compared the clonality results with analysis for JAK2V617F, the mutation was identified in 55% of patients with clonal granulocytes versus 45% of patients with polyclonal granulocytes (p=0.508). JAK2V617F mutations were present in 38.8% of ET patients with clonal granulocytes and in 35.5% of ET patients with polyclonal granulocytes (p=1.000). Regarding PV patients, JAK2V617F mutation was identified in 87.5% of cases with clonal granulocytes and in 72.7% of patients with polyclonal granulocytes (p=0.370). Finally, JAK2V617F mutation was identified in 1/6 (14.3%) AMM patients with clonal granulocytes. The results are shown in the Table. The combination of both techniques allowed to establish myeloid clonality in 42/65 (64.6%) ET, 33/37(89.1%) PV and 7/7 (100%) AMM patients. *Conclusions.* The simultaneous use of JAK2V617F assessment and HUMARA assay in female patients with Ph-negative MPDs increases the detection of myeloid clonality in AMM and ET female patients compared to either technique alone. No correlation was observed between JAK2V617F mutation and HUMARA assay.

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		– Clonality assessment by HUMARA assay –				
		Polyclonal	Clonal	Allelic skewing	Homozygous	Total
ET	JAK2 Wild type	20	11	2	1	34
	JAK2V617F	11	7	5	8	31
	Total	31	18	7	9	65
PV	JAK2 Wild type	3	2		1	6
	JAK2V617F	8	14	3	6	31
	Total	11	16	3	7	37
AMM	JAK2 Wild type		5			5
	JAK2V617F		1		1	2
	Total		6		1	7
TOTAL		42	40	10	17	109

0499

ABERRANT GENE EXPRESSION PROFILE OF CD34+ CELLS IN IDIOPATHIC MYELOFIBROSIS IDENTIFIES A SUBSET OF DISEASE-ASSOCIATED GENES WITH CLINICAL CORRELATES

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Background. Idiopathic myelofibrosis (IM) is a clonal myeloproliferative disorder (MPD) characterized by bone marrow fibrosis, myeloid metaplasia usually accompanied by leukoerythroblastic blood smear, variable degree of pancytopenia or leucocytosis, splenomegaly, and increased number of CD34+ cells in the peripheral blood (PB). A part for the recently described mutation in JAK2 exon 12 in about half of patients, as well as in other MPD, no recurrent chromosomal abnormality nor molecular defect specific for IM has been described to date. *Aims.* As an approach to identify possibly aberrantly regulated genes in IM, we performed a comprehensive transcriptome comparative microarray analysis of normal and IM CD34+ cells. *Methods.* For this purpose, we prepared three pools of >98% pure CD34+ cells from the PB of IM subjects, and two pools from the BM of normal donors, each comprising five subjects. The cDNA was hybridized to an Affymetrix HG-U133A oligonucleotide microarray chip representing 22,283 transcripts. *Results.* Two-hundred eighteen differentially expressed genes were identified; among these, 50 genes that we considered as potentially involved in the pathophysiology of IM were further validated by quantitative RT-PCR. By using class prediction analysis, a set of eight gene markers (CD9, GAS2, DLK1, CDH1, WT1, NFE2, HMGA2 and CXCR4) was employed to successfully recognize normal from IM CD34+ cells. These gene were aberrantly regulated also in the granulocytes of IM, polycythemia vera (PV) and essential thrombocythemia (ET) patients, with some unique patterns; class prediction analysis differentiated IM from normal granulocytes in 100% of cases, while a correct class attribution was obtained in 95% of IM, PV, or ET patients. Altered gene expression was corroborated by the detection of abnormally high CD9 or CD164, and low CXCR4, protein content in CD34+ cells, that characterized IM patients when compared to either normal subjects, PV or ET patients. We speculate that the significant down-regulation of CXCR4 on IM CD34+ cells might

be related to the constitutive mobilization of these cells in the circulation. Abnormal expression of NFE2, HMGA2, and CXCR4 was influenced by the presence of JAK2V617F mutation, unlike WT1, CD9, GAS2, DLK1, and CDH1. There was a direct correlation between expression levels of CD9 and platelet count in IM patients, while DLK1 was inversely related to platelet count. Also, higher WT1 expression levels identified IM patients with more active disease, as evidenced by elevated CD34+ cell count and higher severity score. *Conclusions.* According to the currently employed clinical-biological parameters, the differential diagnosis among the chronic myeloproliferative disorders may still remain uncertain in a substantial proportion of cases, owing their phenotypic overlapping; therefore, the possibility to use a defined set of molecular markers to approach the diagnosis of IM is of clinical relevance, and has potential diagnostic application. Moreover, determination of WT1 expression levels in IM patients might be suitably employed as the first molecular marker of disease activity and, perspectively, prove useful also to evaluate response to therapy.
On behalf of the MPD Consortium)

Antibodies in the treatment of non-Hodgkin's lymphoma

0500

HUMAX-CD20, A NOVEL FULLY HUMAN ANTI-CD20 MONOCLONAL ANTIBODY: RESULTS OF A PHASE I/II TRIAL IN RELAPSED OR REFRACTORY FOLLICULAR NON-HODGKINS LYMPHOMA

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Background. The fully human monoclonal IgG1 antibody HuMax-CD20 targets a novel epitope of the CD20 molecule on B-cells. HuMax-CD20 stops growth of engrafted B-cell tumors in SCID mice more efficiently and exerts stronger complement activation than Rituximab. HuMax-CD20 kills Rituximab-resistant cells expressing low levels of CD20. *Aims.* The objective of the present trial is to establish the safety, efficacy and the pharmaco-kinetics of HuMax-CD20 in patients with relapsed or refractory follicular lymphoma grade 1-2. *Methods.* Data are presented from a recently completed, open label, dose-escalation, multicenter clinical trial in patients with relapsed or refractory CD20+ follicular non-Hodgkin's lymphoma. Four cohorts of 10 patients were treated with 4 weekly i.v. infusions of 300, 500, 700 or 1000 mg. The endpoints include adverse events, centrally reviewed CT verified tumor response according to the Cheson criteria, B-cell depletion, pharmacokinetics and progression free survival. *Results.* Fourty patients have been treated. Mean age was 57 years; median number of prior treatment regimens was 2; 15 patients were previously treated with Rituximab. Rapid, efficient and sustained peripheral B-cell depletion was observed in all dose groups. No dose limiting toxicity has been reported. Only 8 short lasting episodes of grade 3 CTC were observed. Hematological toxicity was low and confined to 6 events of grade 1 neutropenia; no cases of thrombocytopenia were reported. The following pharmacokinetic parameters were derived (medians per dose group): Cmax 129, 185, 380 and 610 μg/mL, $T^1/_2$ 447, 245, 322 and 621 hr, Cl 9, 19, 10 and 7 mL/hr/kg and AUC 75000, 51000, 185000 and 326000 hr μg/mL, for the 300, 500, 700 and 1000 mg dose groups, respectively. No correlation between pharmacokinetics and response was found. Objective responses (CR, CRu, PR) have been evaluated in 37 patients and were obtained in all 4 dose groups; 4 CR + 1 CRu/8 (300 mg), 1CR + 2 PR/9 (500 mg), 2PR/10 (700 mg) and 1 CRu + 4 PR/10 (1000 mg). Objective responses were achieved in 9 of 14 (64%) evaluable patients previously treated with Rituximab, i.e. 3CR, 1 CRu and 5 PR. In total 18 patients showed stable disease; progression was observed in only 3 patients. Based on Kaplan Meier estimates, the median time to progression for all patients was 267 days (95% CI 133-372 days). The median time to progression for responders and the median duration of response have not yet been reached. *Conclusions.* This final analysis demonstrates a favourable safety profile and encouraging efficacy of HuMax-CD20 in patients with follicular NHL. Objective responses were achieved in all dose groups with response rates up to 63%, including a 64% response rate in patients previously treated with Rituximab. The median time to progression for responders has not yet been reached.

0501

ALEMTUZUMAB, FLUDARABINE, CYCLOPHOSPHAMIDE, AND DOXORUBICIN (CAMPATH-FCD): AN EFFECTIVE FIRST-LINE TREATMENT IN PERIPHERAL T-CELL LYMPHOMAS

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Background. Peripheral (mature) T-cell lymphomas (PTCL) represent a group of lymphoma entities with an unfavourable outcome after treatment with CHOP or CHOP-like regimens. *Aims.* The purpose of the study was to investigate the feasibility of a combination of the monoclonal antibody alemtuzumab with chemotherapy consisting of fludarabine, cyclophosphamide and doxorubicin. *Methods.* Patients were treated with alemtuzumab 3, 10, 30, 30 mg, days 1-4, fludarabine 25 mg/m^2 days 2-4, cyclophosphamide 600 mg/m^2 day 3, and doxorubicin 50 mg/m^2 day 4. Included were patients with primary diagnosis, with first relapse, or with primary refractory disease, excluded were patients with primary cutaneous T-cell lymphomas and ALK-positive large cell anaplastic T-cell lymphomas. *Results.* So far, 33 patients have been included, 26 are evaluable for response and toxicity: 12 patients with PTCL-NOS, 9 with AILD, two with ALK negative ALCL, one with enteropathy-associated T-cell lymphoma, one with NK-cell lymphoma, and one with T-PLL. 15 patients were enrolled with primary diagnosis and 11 patients in relapse. The median age was 58 years (range 21-77); 71% of the patients had an international prognostic index intermediate high or high. In patients with primary diagnosis the CR rate was 67% (10/15), three patients were primary progressive, and two patients dropped out because of treatment associated complications. 9 of the responding patients are in ongoing CR at 2+, 5+, 6+, 13+, 14+, 17+, 20+, 26+, and 28+ months, respectively. The patient with T-PLL relapsed after being in CR for 23 months. In the group of relapsed or refractory patients two CR and two PR (36% overall response) were observed. The main toxicity was leukocytopenia (64% grade III and IV of all evaluable treatment cycles), other grade III and IV toxicities included anemia (17%), thrombocytopenia (33%), infections (14%), pruritus/skin reactions (9%), nausea/emesis (6%), mucositis, and cardiac toxicity (5%, two patients with relapsed disease after pre-treatment with CHOP-like regimens developed severe heart failure). 11 (42%) patients reactivated CMV, however, 9 without developing CMV-related disease. *Conclusions.* The combination is an effective first-line regimen for peripheral T-cell lymphoma, however, regarding the general outcome a longer follow-up period of a larger patient population is required. Because the results were not convincing in relapsed disease and because of two heart failures in this group, the study was closed for relapsed and refractory patients, but is ongoing for first-line treatment of peripheral T-cell lymphomas.

0502

GALIXIMAB (ANTI-CD80 MONOCLONAL ANTIBODY) IN COMBINATION WITH RITUXIMAB FOR RELAPSED OR REFRACTORY, FOLLICULAR NHL: RESULTS OF A PHASE II STUDY

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Background. Galiximab is a monoclonal antibody that targets CD80, a costimulatory molecule that is constitutively expressed on the surface of follicular and other lymphomas. Modest single-agent clinical activity and tolerability were demonstrated in a Phase I study with galiximab in relapsed or refractory, follicular NHL (ORR=11%). Preclinical studies have shown that galiximab and rituximab may exhibit at least additive activity in lymphoma models, supporting the rationale for a combination study. *Aims.* A Phase I/II study (Study 114-21) evaluating galiximab + rituximab in relapsed or refractory, follicular NHL. Study objectives were to determine safety, pharmacokinetics, and efficacy of the combination regimen. Here we report final results from the Phase II part of the study. *Methods.* Patients received galiximab (500 mg/m^2 qwk x 4) with a standard course of rituximab (375 mg/m^2 qwk x 4). Rituximab refractory patients (no response or a response with TTP <6 months) were excluded. International Workshop Response Criteria were used to evaluate response. *Results.* Sixty-four patients received treatment. The median follow-up is 20.4 months. Median age at study entry was 59 yrs. Eighty-eight percent of patients were Stage III/IV, with FLIPI low (27%), intermediate (39%), or high (34%) risk groups. All patients had received at least 1 prior lymphoma therapy; 42% were rituximab naïve. Galiximab infusions were delivered over 1 hr and were well tolerated. No DLTs were reported. Sixty one (95%) patients experienced study related AEs; the most common were lymphopenia (44%), leucopenia (38%), fatigue (38%), neutropenia (23%), and chills (23%). An ORR of 64% was observed: 17% CR, 16% CRu, and 31% PR. The median PFS was 12.1 months. Combination therapy did not appear to alter pharmacokinetics. The mean serum half life was 25.7 days for galiximab and 24 days for rituximab. These results were retrospectively compared with 3 historical studies of follicular NHL patients treated with a standard course of rituximab monotherapy. Baseline characteristics were similar; however, there was a higher incidence of rituximab-naïve patients in the rituximab monotherapy group (77%) compared with galiximab + rituximab (42%). The toxicity profile of the combination regimen was similar to that observed in the single agent rituximab studies. However, the median PFS was longer in the galiximab + rituximab group (12.1 months) than in the historical rituximab monotherapy group (9.4 months). In a subset analysis of rituximab-naïve patients, the difference in PFS was even more pronounced: 15.4 months in the galiximab + rituximab group vs. 9.4 months in the rituximab monotherapy group. *Conclusions.* These results suggest that galiximab can be safely combined with a standard course of rituximab, produce promising response rates, and may potentially extend PFS in patients with relapsed or refractory, follicular NHL. A Phase III, randomized, double-blind study is planned.

0503

MT103 (ANTI-CD19 X ANTI-CD3 BITE) INDUCES B CELL DEPLETION, CLEARANCE OF BONE MARROW INFILTRATION AND CLINICAL RESPONSES IN HEAVILY PRE-TREATED NHL PATIENTS: FIRST DATA FROM DOSE-ESCALATION STUDY MT103-104

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Background. MT103 is an anti-CD19/anti-CD3 bispecific single-chain antibody construct. Preclinical studies have shown that low picomolar concentrations of MT103 can redirect unstimulated human T cells against CD19-positive human B lymphoma and normal B cells leading to their efficient lysis. MT103 is further characterized by mounting a polyclonal T cell response, an activity at very low effector to target cell ratios, and an apparent independence of T cell costimulation. Corticosteroids were co-administered as anti-inflammatory agents. Initial findings have shown that MT103 has an estimated half-life of approximately two hours. Here we describe a continuous infusion regimen. *Aims.* The MT103-104 study was set up to explore the safety and tolerability of increasing doses of MT103 given as continuous infusion. Additional objectives include the assessment of MT103 continuous infusion PK profile, the collection of pharmacodynamic data and the observation of clinical efficacy. *Methods.* Patients with relapsed indolent NHL were included according to a classical 3+3 dose escalation design with initial MT103 doses of 0.5 µg/m^2/24h with initial steroid coverage. Safety and tolerability were assessed by CTC-AE criteria and dose escalation was only allowed after a data review committee (DRC) concluded safety of the previous dose with a DLT observation period of 14 days. Biological activity was monitored by investigating levels of systemic cytokines using specific ELISAs and by quantification and characterisation of peripheral immune cell subsets via FACS analysis. After 4 weeks of MT103 treatment, a control CT scan was performed. If patients were at least stable

according to standardized Cheson criteria (reviewed by central radiology), an additional 4-week cycle of MT103 was offered to the patients. *Results.* As of today, 19 patients with a median number of 4 previous chemo-/immuno therapies have been included into MT103-104. During dose-escalation from DL1 (0.5 µg/m²/24h) up to DL3 (5 µg/m²/24h) no dose-limiting toxicity was observed, and AEs were generally moderate. At DL4 (5 µg/m²/24h on the first day, 15 µg/m²/24h as maintenance dose), 7 patients were treated with 2 patients receiving less than 14 days of treatment. One patient experienced elevation of liver enzymes up to CTC grade 3 after 2 weeks, which recurred upon re-administration of MT103; and 1 patient experienced confusion and disorientation on the second day of treatment. Depletion of circulating B (lymphoma) cells by end of the MT103 infusion was observed in 9 of 15 evaluable patients (with treatment for >2 weeks and B cells detectable in peripheral blood prior to MT103 infusion) with a dose-dependent increase in frequency that reached 100% depletion at DL4. At DL4, 3 of 7 patients had significant bone marrow (BM) infiltration (>10%) with 1 patient showing reduction of and 2 patients showing complete disappearance of lymphoma cells in BM. Best overall tumour response in the 14 evaluable patients (with treatment for >2 weeks and scanning of all involved areas) was 1 CR (at DL4), 2 PR (at DL4), 1 MR, 7 SD and 3 PD. *Summary*. These preliminary results observed in indolent NHL patients clearly indicate single agent biological and clinical activity of MT103.

0504

RITUXIMAB ADMINISTERED WITH MEGA-CHOEP HIGH-DOSE CHEMOTHERAPY AND AUTOLOGOUS TRANSPLANTATION OF HEMATOPOIETIC STEM CELLS IMPROVES OUTCOME FOR PATIENTS WITH DLBCL AND HIGH LDH

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Background. The German High-Grade Lymphoma Study Group (DSHNHL) performed a series of phase II studies evaluating the Mega-CHOEP programme in younger patients (< 60 yrs) with aggressive lymphoma and elevated LDH. MegaCHOEP consists of 4 courses of intermediate to high-dose chemotherapy (cumulative doses at highest dose level: C: 19.5 g/m², H: 280 mg/m², O: 8 mg, E: 5.04 g/m² and P: 2000 mg) followed by autologous SCT after treatment courses 2-4 (Glass *et al.*, BLOOD 2006 and Schmitz *et al.* CANCER 2006). *Aims.* To compare the feasibility, safety, and efficacy of MegaCHOEP with and without adding 6 infusions of Rituximab (R: 375 mg/m²) on days 1, 14, 35, 56, 77, and 98 of the treatment programme. *Methods.* The time to treatment failure (TTTF) and overall survival (OS) of all patients with CD20 positive diffuse large B-cell lymphoma (DLBCL) who were treated with Mega-CHOEP, dose level 3 with R (DL3+R) or without R (DL3) were compared using univariate and multivariate analyses. *Results.* 51 patients were enrolled at DL3 and 89 patients were enrolled at DL3+R. There were no significant differences in stem cell yield, non-hematologic toxicity, or hematopoietic recovery between patients given MegaCHOEP with or without R. Univariate analysis of TTTF and OS showed significant improvement with the addition of R. With a median follow-up of 2.6 years (MegaCHOEP + R) and 4.5 years (MegaCHOEP) OS was 79% with and 55% without R. A multivariate analysis adjusting for the International Prognostic Index (IPI) showed a superior OS for patients given R (relative risk for failure of 2.5 [p=0.034]). The relative risk for TTTF was 2.4 (p=0.023) when MegaCHOEP patients treated without or with R were considered. *Summary/Conclusions.* We conclude that in patients with DLBCL the addition of R to dose escalated CHOP plus Etoposide (MegaCHOEP) significantly improves OS and TTTF without adding significant toxicity. The DSHNHL is currently running a prospective randomized trial comparing R-MegaCHOEP with 8 courses of R-CHOEP given every 2 weeks in young high-risk patients with aggressive lymphoma.

Cancer genetics and cytogenetics in myeloid diseases

0505

GENE EXPRESSION PROFILING FOR MOLECULAR SUBCLASSIFICATION OF LEUKEMIA: INTERIM ANALYSIS OF THE INTERNATIONAL MULTI-CENTER STUDY (MILE) ON 1437 PATIENTS

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Background. Microarray analysis can identify differentially expressed genes associated with distinct clinical and therapeutically relevant classes of both pediatric and adult leukemias. *Aims.* The MILE (Microarray Innovations in Leukemia) study has started in 11 centers (European Leukemia Network (ELN): 7, USA: 3, Singapore: 1). MILE compares the accuracy of gene expression profiles of 16 acute and chronic leukemia subclasses, MDS, and non-leukemia as control group to current routine diagnostic workup. *Methods.*In a pre-phase each center was trained on the identical microarray protocol and used the same laboratory equipment and reagents for target preparation and analysis on Affymetrix HG-U133 Plus 2.0 microarrays. Two cell lines (MCF-7, HEPG2) were tested. In parallel, each center prepared total RNA from cell lysates of three leukemia patients (AML with t(8;21), CML, and CLL) and processed them with replicates. After successfully passing proficiency testing, the centers started to analyze prospectively 2000 leukemia samples as next step of MILE. *Results.* The pre-phase demonstrated a very high intra- and inter-laboratory comparability among the participating centers. In detail, unsupervised analyses of a total of n=175 analyses accordingly grouped each sample type in unique clusters. Remarkably, the replicates of the leukemia samples demonstrated squared correlation coefficients of gene expression ranging between 0.930 and 0.997 (median=0.984) for the CML, between 0.936 and 0.998 (median=0.983) for the CLL, and between 0.940 and 0.999 (median=0.988) for the AML with t(8;21) sample. Here we present for the first time classification results of a first series of n=1437 tested patients from the 11 centers that were included in a training data set to form linear classifiers for all 18×(18 - 1) / 2 = 153 class pairs. The average cross-validation accuracy of this training data set is 89.4%. This classifier was further tested on two independent patient cohorts. In a first independent cohort (HG-U133 Plus 2.0, n=105) 89.5% classification accuracy were achieved. In a second independent cohort (n=1,094), analyzed previously in two centers on HG-U133A/B microarrays, 83.5% classification accuracy were achieved. In detail, 136 out of 139 (97.8%) chronic leukemia samples (CML or CLL) are classified fully in agreement with standard diagnostic procedures. For acute leukemia subtypes 767/904 (84.8%) are classified correctly. In the MDS group (n=81) miscalls occur both in the distinction between MDS and AML with normal karyotype or cytogenetically so-called other aberrations as well as between and MDS and non-leukemia. Interestingly, an AML-like signature can be found in MDS samples correlating with IPSS >1.5. These samples are currently further investigated regarding a potential later progression into full-blown AML. *Conclusions.* This international multi-center study demonstrates a very high inter- and intralaboratory reproducibility of microarray analyses. Moreover, a first series of 1437 leukemia patients was successfully analyzed and classified with high accuracy. Data will be used to design a new custom format microarray dedicated to further develop the application of gene expression profiling for diagnosis and subclassification of leukemia.

0506

TRISOMY 8 IS ASSOCIATED WITH A HIGHER EXPRESSION OF A SUBSET OF GENES LOCATED ON CHROMOSOME 8 DETERMINED BY THE ACCOMPANYING GENETIC ABNORMALITIES

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Background. Trisomy 8 is the most frequently observed trisomy in AML occurring as a sole karyotype abnormality or in addition to other chromosome aberrations. 2. *Aim.*It was the aim of this study to analyze the impact of trisomy 8 on the expression of genes located on chromosome 8 in different AML subgroups. 3. *Methods.* Gene expression analyses were performed in a total of 567 AML cases.The following 14 subgroups were analyzed: +8 sole (n=19), +8 within a complex aberrant karyotype (n=11), +8 with t(15;17) (n=7), +8 and inv(16) (n=3), +8 with t(8;21) (n=3), +8 and 11q23/MLL (n=8), and +8 with other abnormalities (n=10). These were compared to 200 AML with normal karyotype and the following subgroups without trisomy 8: complex aberrant karyotype (n=73), t(15;17) (n=36), inv(16) (n=46), t(8;21) (n=37), 11q23/MLL (n=37), and other abnormalities (n=77). 4. *Results.* A significant higher mean expression of genes located on chromosome 8 was observed in subgroups with +8 in comparison to their respective control groups. A varying number of significantly higher expressed genes was identified in all comparisons. No gene was significantly overexpressed in all comparisons. No distinct gene expression pattern was identified allowing the identification of cases with trisomy 8. Therefore, the gain of chromosome 8 leads to a higher expression of genes located on chromosome 8. However, no consistent pattern of genes was identified which shows a higher expression in all AML subtypes with trisomy 8. 5. Summary / *Conclusions.* This data suggests that trisomy 8 rather provides a platform for a higher expression of chromosome 8 genes which are individually upregulated by the respective primary genetic abnormalities. Therefore, trisomy 8 in AML determines no specific disease characteristic but is a disease modulating secondary event.

0507

JAK2 AND FLT3 MUTATION SCREENING IN 256 PATIENTS WITH AML/MDS AND A NORMAL KARYOTYPE. THE PRESENCE OF V617F CORRELATES WITH A HIGHER FREQUENCY OF CRYPTIC GENETIC ABERRATIONS

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AML and MDS are clinically and molecularly heterogeneous diseases. Karyotype is one of the most important prognostic factors. Effective risk stratification is especially difficult for patients with normal karyotype, which account for more than 40% of all cases. In AML, the clinical outcome of patients with normal cytogenetics can be predicted by the presence or absence of mutations or by changes in the expression of specific genes. Mutations in FLT3 are detected in 30% of AML and in 3-5% of MDS, and are associated with unfavorable prognosis. The V617F activating mutation of JAK2 has been recently described as a common event in MPD, and has been found in a small number of patients with either AML or MDS. Although there are few studies, the incidence of JAK2 mutation seems to be similar in both groups (3%). However, apart from CMML (5.8%), there are no data about its relationship with either specific MDS subgroups or with the karyotype. More still, a detailed correlation between mutant JAK2 and FLT3 has yet been fully addressed. In this study we investigated the incidence of JAK2 and FLT3 mutations in 176 patients with AML and 80 MDS, with normal karyotype, and its relationship with cryptic genetic aberrations analyzed through a SNP array platform. V617F genotyping was performed by ARMS as previously described (Jones el al., 2005). All cases tested positive were confirmed by sequencing. A high resolution 50K SNP array (Affimetrix) was used to analyze 14 patients. We found the mutation V617F in 10 cases: 6 AML (3.4%) and 4 MDS (5%), confirming the low incidence of this mutation in AML and MDS. The frequency in the FAB subgroups was 2% in M1 (1/50), 10.8% in M2 (5/46), 3.5% CMML (1/28), and 11% RAEB/RAEBT (3/27). As expected, 30% of AML had FLT3 mutations, and only 2.5% of the MDS patients (2/80). Of the 28 CMML patients analyzed, one had V617F (3.5%), and 2 ITD-FLT3 (7.7%), suggesting that it would be interesting to analyze FLT3 mutations in MPD patients with no JAK2 mutations. As previously reported, the SNP array technology permits the simultaneous analysis of copy number and allelotype data. Using the 50K SNParray, cryptic genetic aberrations were identified in the 14 patients analyzed. We found 13 recurrent changes. Five cases had regions of uniparental disomy (35%), confirming the importance of this mechanism in AML. Interestingly, the median of aberrations in the JAK2 mutated cases was higher than in those not mutated (6 vs 2.5), and we detected 3 recurrent amplifications in the mutated cases. In conclusion, we found that more than 30% of the cases with normal karyotype had regions of LOH by UPD. The SNP array technology could be a useful method to define subgroups in patients with normal karyotype, and we found a higher frequency of cryptic aberrations in cases with V617F. The incidence of the mutation V617F is low in both AML and MDS, although prospective studies are needed to confirm the definitive role of these mutations in the patients prognosis

0508

GENETIC PATHWAYS OF THERAPY-RELATED MDS AND AML (T-MDS/T-AML)

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Background. t-MDS/t-AML may serve as a model for leukemic transformation. *Aims.* to relate cytogenetic characteristics to mutation of eleven different genes in t-MDS/t-AML. *Methods.* 89 patients with t-MDS and 51 patients with t-AML were studied by standard *Methods. Results.* Eight different genetic pathways were identified. Pathway I included patients with t-MDS and 7q/-7 but normal chromosomes 5 and without balanced translocations. Point mutations of AML1 and methylation of the p15 promotor were common in this pathway. Pathway II included patients with t-MDS and 5q/-5 with or without abnormalities of chromosome 7. In this pathway 77% presented p53 mutations often combined with deletions of 17p, complex karyotypes and complicated chromosome rearrangements. Sometimes amplification of 11q23 or 21q22 was observed. Most patients in pathway I and II had received alkylating agents. Pathway III included patients with t-AML and translocations to 11q23. RAS and BRAF mutations were common in this pathway. Pathway IV included patients with balanced chromosome aberrations involving 21q22 or 16q22. Except for cases with t(3;21) these patients presented as t-AML. Patients with involvment of 21q22 often had additional 7q/-7 and occasionally c-KIT or PTPN mutations. Pathway V included patients with t-AML and translocations to 17q12. They sometimes presented FLT3-ITD. Pathway VI included cases of t-MDS or t-AML and balanced translocations to 11p15 and rearrangement of the NUP98 gene. Most patients in pathway III-VI had previously received topoisomerase II inhibitors. Pathway VII included patients with a normal karyotype often presenting as t-AML and 50% had mutations of FLT3, RAS or AML1, frequently with mutation of the last two genes in combination. Mutation of JAK2 was observed in only two atypic cases of t-MDS in this pathway. Pathway VIII included patients with atypic chromosome arrangements and only 2/20 in this pathway presented RAS mutations. A significant association was observed between mutations of genes in the RTK/RAS-BRAF signalling pathway (n=33) and mutation or rearrangement of genes for putative transcription factors (n=49), so called class I and class II mutations, as 18 patients, 15 belonging to pathways III-V and VII, presented both types of mutation (p=0.012, Fishers exact test, two sided). *Conclusions.* classification of t-MDS/t-AML in different genetic pathways is supported and an association between class I and II mutations is confirmed.

0509

NEW STRATEGY TO IDENTIFY TUMOR SUPPRESSOR GENES IN ACUTE MYELOID LEUKEMIA

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Background. Retroviral integration mutagenesis in mice is a powerful tool to discover novel genes involved in the development of leukemia. Using the Graffi 1.4 murine leukemia virus (Gr-1.4 MuLV), we identified candidate disease genes of acute myeloid leukemia (AML) (Erkeland *et al.*, J Virol. 2004: 78; 1971-80). Recently, we reported that genes adjacent to the virus integration site (VIS), so-called VIS genes, contribute significantly to gene expression profiles of distinct subgroups of human AML, supporting the importance of deregulation of VIS genes in the pathogen-

esis of AML (Erkeland *et al.,* Cancer Res. 2006: 66; 622-6). Because MuLV preferentially, albeit not exclusively, integrate in the 5' promoter region of genes, it is generally assumed that expression of VIS genes is most frequently increased due to the transcription enhancing activities of the viral LTR. However, CpG islands within the LTR are potential target for *de novo* methylation, which could form the initiating event for silencing due to methylation spreading. This would imply that retroviral mutagenesis screens could also be used for identification of potential tumor suppressor or haplo-insufficient genes. *Aim*. To determine whether and to what extent proviral LTR sequences are methylated in Gr-1.4 MuLV-induced myeloid leukemia; (2) To investigate whether methylated LTR sequences can be used to identify (new) potential tumor suppressor genes; (3) To establish the significance of these genes for human AML. *Methods.* A methylation sensitive quantitative PCR (Q-PCR) was developed to determine the ratio between methylated and unmethylated LTR in the different tumor samples (n=81). Samples that showed high levels of methylated LTR were used to identify methylated VIS flanking genes. To this end, we developed a novel method in which methylated DNA immunoprecipitation (MeDIP) with anti-5-methylcytidine (a5-mC) antibody is combined with inverse PCR (iPCR). *Results.* The methylation sensitive Q-PCR showed a marked heterogeneity of LTR methylation status among different tumor samples. Distinct methylation categories were defined: high (n=7), medium-high (n=15), medium (n=12), low (n=20) and none (n=27). Enrichment of LTRs after MeDIP with a5-mC was found in 25/34 samples. As expected, MeDIP on normal hematopoietic tissues was negative for LTR, but positive for the methylation imprinted gene H19. MeDIP/iPCR resulted in 1 to 7 bands per tumor sample. These gene products include known suppressor genes such as Smad1 and Mad1-like, as well as a number of genes with as yet poorly characterized roles in cancer. *Summary/conclusions.* We present a new strategy to identify tumor suppressor genes in AML. The marked variability in DNA methylation of VIS in different tumor samples indicates that most viral integrations occur in non-methylated parts of the DNA, otherwise the DNA methylation of VIS in different tumor samples would be more equal. The potential tumor suppressor genes in these regions, which may be silenced through methylation spreading, will be identified by direct PCR strategies in combination with bisulfite treatment. To test the relevance of these genes for clinical disease, their expression will be analyzed in a large cohort of AML patients, of which gene expression profiles are already available.

Thrombosis

0510

ORAL CONTRACEPTIVE USE, THROMBOPHILIA AND THEIR INTERACTION FOR ISCHEMIC STROKE IN YOUNG WOMEN

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Background. Oral contraceptive use is a risk factor for ischemic stroke in young women. While hyperhomocysteinemia increases the risk of the disease, the role of inherited thrombophilia is still uncertain. Little data exists on the interaction between such risk factors and the risk of ischemic stroke in young women. *Aims.* To assess the interaction between thrombophilia and oral contraceptive intake in determining the risk of ischemic stroke in young women. *Methods.* One-hundred and five women with a first ischemic stroke at an age less than 45 years and 293 healthy controls were investigated for the presence of thrombophilia due to factor V Leiden, prothrombin G20210A mutation, antithrombin, protein C and protein S deficiency, and hyperhomocysteinemia. The presence of oral contraceptive use was recorded. Fifty-five women had a stroke of undetermined origin. *Results.* Oral contraceptives were associated with an increased risk of ischemic stroke (odds ratio 2.3, 95%CI 1.4-3.8) in the first 6-18 months of use. The risk of ischemic stroke was also higher in patients with hyperhomocysteinemia (odds ratio 3.5, 95%CI 1.9-6.4), in those with factor V Leiden (odds ratio 2.6, 95%CI 0.8-8.0), but not in those with prothrombin G20210A (odds ratio 0.9, 95%CI 0.1-11.2). After stratification for the presence of oral contraceptive use and thrombophilia due to factor V Leiden or hyperhomocysteinemia, the odds ratio for ischemic stroke in women with both risk factors was 12.9 (95%CI 1.3-133.7) for factor V Leiden and 9.2 (95%CI 2.5-33.5) for hyperhomocysteinemia. No increased risk was observed when oral contraceptive use and prothrombin G20210A were present together. The risk associated with oral contraceptive use, hyperhomocysteinemia, or both, was more pronounced for stroke of undetermined than determined etiology. *Conclusions.* The use of oral contraceptives is associated with a 2-fold increased risk of ischemic stroke use. This risk quintuplicates in the presence of hyperhomocysteinemia or factor V Leiden. These findings should be taken into account for thrombophilia screening after an ischemic stroke and for individual decision making when prescribing oral contraceptives.

0511

VENOUS THROMBOEMBOLISM A METABOLIC DISEASE?

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Background. Deep venous thrombosis (DVT) and pulmonary embolism (PE) are amongst the most common disorders in developed countries. Only in about 50% of patients with a history of unprovoked venous thromboembolism (VTE) a genetic or acquired risk factor can be specified. Experimental, epidemiological and clinical studies indicate an association between serum lipids and VTE. Hyperlipidemia and overweight may play a role in the development of VTE by influencing the homeostasis of the clotting and fibrinolytic system and could thereby induce a hypercoagulable state. *Aim.* The aim of our present study was to elucidate a possible association of lipids and overweight with VTE in high risk patients, who suffered from objectively confirmed recurrent VTE. *Methods.* We conducted a case-control study to analyse the relationship between serum lipids and the risk of VTE. Outpatients with a history of objectively confirmed recurrent VTE, who had at least one event of an unprovoked DVT or PE, were recruited from 01/2005 to 11/2005. Age and sex-matched healthy individuals served as controls. Venous blood samples were obtained after overnight fasting for serum lipid determinations (total cholesterol, low density lipoprotein (LDL), high density lipoprotein (HDL) and triglycerids). Height (m) and weight (kg) were recorded and body mass index (BMI) was calculated (kg/m^2). Hyperlipidemia was diagnosed, when serum cholesterol level was over 200 mg/dL, triglyceride level over 172 mg/dL or cholesterol/HDL-quotient > 4. A BMI above 24.99 kg/m^2 characterized overweight. Mann-Whitney-U test was carried out to compare the groups. Univariate logistic regression analyses were applied to calculate odds ratios and the 95% confidence interval. *Results.* Hundred-sixteen patients (53 female / 63

male; mean age 56 ±12 yrs) with a history of recurrent VTE and 129 age and sex-matched controls (66 female / 63 male; mean age 53 ±11 yrs) were enrolled. Patients showed a significantly higher BMI than controls (median (Md) 27.45 kg/m^2 vs 25.78 kg/m^2, p=0.032). Total cholesterol (Md = 233 mg/dL vs. 230 mg/dL, p=0.22) and LDL (Md = 147 mg/dL vs. 141 mg/dL, p=0.074) serum levels were not significantly different. HDL levels were significantly lower in patients (Md = 56 mg/dL vs. 60 mg/dL, p=0,035) and the cholesterol/HDL-quotient significantly higher (Md = 4.24 vs. 3.66, p=0.001). Triglyceride levels also differed significantly (Md = 135 mg/dL vs. 111 mg/dL, p=0,006). Estimated odds ratios for VTE were 1.86 (95% CI, 1.08-3.19; p=0.024) for overweight, 1.32 for hypercholesterolemia (95% CI, 0.72 ' 2.40; p=0.37), 1.85 (95% CI, 1.04 ' 3.28; p=0.037) for hypertriglyceridemia and 2.12 for cholesterol/HDL-quotients > 4 (95% CI, 1.25-3.60; p=0.006). *Summary/Conclusion.* Overweight, hypertriglyceridemia and a cholesterol/HDL-quotient > 4 increase the risk for VTE by almost doubling it. Therefore, besides the well known genetic factors, we suspect a possible relationship between environmental factors such as nutrition or physical activity and development of VTE.

0512

THE PERSISTANCE OF RESIDUAL VEIN THROMBOSIS, AFTER AN EPISODE OF DEEP VEIN THROMBOSIS, AND THE RISK OF NEW OVERT CANCER AND CARDIOVASCULAR DISEASE

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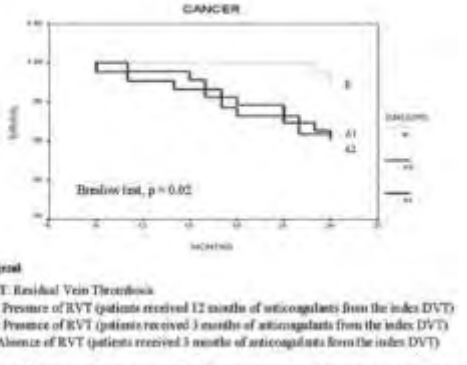
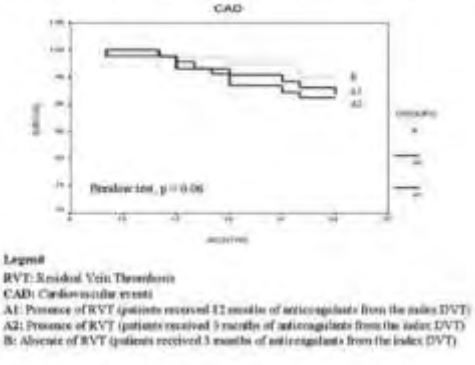
Background. We have recently demonstrated that the presence of Residual Vein Thrombosis (RVT), UltraSonography (US)-detected at the 3rd month after an episode of Deep Vein Thrombosis (DVT) of the lower limbs, is an independent risk factor for developing recurrent Venous Thromboembolism (VTE). The management of DVT patients by detection of RVT may, therefore, represent a simple and reproducible method for establishing the individual risk of recurrence and for tailoring the optimal duration of Oral Anticoagulants (OA) (Siragusa S *et al.* Blood 2003;102(11):OC183a). At the present, it is unknown whether RVT may also identify patients at increased risk for cancer and/or cardiovascular disease (CD). Objective of the study. In patients with DVT of the lower limbs, we conducted a prospective study for evaluating the correlation between RVT and the risk of new overt cancer and/or CD. Materials and

Figure 1: Relationship between RVT and subsequent Cancer



Legend
RVT: Residual Vein Thrombosis
A1: Presence of RVT (patients received 12 months of anticoagulants from the index DVT)
A2: Presence of RVT (patients received 3 months of anticoagulants from the index DVT)
B: Absence of RVT (patients received 3 months of anticoagulants from the index DVT)

Figure 2: Relationship between RVT and subsequent Cardiovascular Event



Legend
RVT: Residual Vein Thrombosis
CAD: Cardiovascular events
A1: Presence of RVT (patients received 12 months of anticoagulants from the index DVT)
A2: Presence of RVT (patients received 3 months of anticoagulants from the index DVT)
B: Absence of RVT (patients received 3 months of anticoagulants from the index DVT)

Methods. Consecutive patients, with an episode of idiopathic or provoked DVT, were evaluated after 3 months from the index DVT. The presence/absence of RVT was detected and patients managed consequently; in those with RVT, OA was continued for 1 year while in those without RVT, OA was discontinued. The incidence of VTE recurrence, overt cancer and new CD was evaluated over a period of almost 3 years after the index DVT. Survival curves (Kaplan-Mayer) and related Breslow test have been used for statistics. *Results.* Three-hundred fourty-five patients were included in the analysis. The results are listed in the figures. The incidence of recurrent VTE and new overt cancer was statistically lower in patients without RVT than in those with RVT; no significant differences were found in the incidence of new CD. These data are applicable in patients with idiopathic or provoked index DVT. In patients with RVT, the advantage of prolonging anticoagulation for 12 months is lost at the end of the treatment. *Conclusions.* This is the first study evaluating the relationship between US-detected RVT and the risk of developing cancer and CD; RVT presence, at 3rd month from the index DVT, is an independent risk factor for recurrent VTE and indicates patients at risk for new overt cancer. This risk remains over a period of almost 3 years, independently whether index DVT was idiopathic or provoked. In these patients, the advantage of indefinite anticoagulation should be assessed in properly designed study.

0513

REGULATION OF PROTEIN S EXPRESSION BY SEX HORMONES

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Background. The anticoagulant Protein S (PS) is coded for by the PROS1 gene and serves as a co-factor to APC inactivation of FVa and FVIIIa. Previous studies have shown a reduction in circulating PS levels in response to increasing oestrogen (E2) levels resulting in an increased thrombotic risk. This relationship is evident in women who are pregnant or are using oral contraceptives (OCs). To date, a mechanism to describe this relationship at the molecular level has not been elucidated. We have identified a potential oestrogen response element (ERE) spanning nucleotides -350 to -367 within the promoter region of PROS1. *Aims.* To devise a method that quantitatively measures the expression of the PROS1 promoter and use it to study the effect of E2. *Methods.* Using an EGFP expression vector, clones containing 950bp of the PROS1 promoter up to, but not including the ATG codon and which included the ERE motif, were transiently transfected into the breast carcinoma cell line, MCF-7. Levels of EGFP were measured by flow cytometry following exposure to E2; with and without excess oestrogen receptor α (ER_). Progestins form the second component of most OCs, therefore the same experiment was performed with progesterone (P4). Co-transfection's with excess progesterone receptor's A and B (PRA and PRB) were also assessed. *Results.* Reflecting clinical observations the expression of the PROS1 promoter fragment decreased in response to E2 and was further reduced in the presence of excess ER_. Interestingly, the opposite was seen in response to P4. Up-regulation via P4 was further increased in the presence of excess PRB, but not PRA. *Summary/Conclusions*. These results show that PROS1 promoter expression is reduced in the presence of E2. The down-regulation is enhanced by ER_, suggesting that the effect is mediated via an ER_ dependent mechanism. However, the promoter region is also responsive to P4 which up-regulates expression in what appears to be a mechanism involving PRB. The opposing effects seemingly counterbalance each other. Based on these results the attention given to the oestrogen component of OCs may not be as important as the progestin component. It is the progestin that varies between different OC preparations and not the oestrogen which is predominantly ethynyl oestrodial. Thus, the increasing evidence that 3rd generation OCs represent a greater thrombotic risk when compared to 2nd generation formulations, could be more about the types of progestins used in the 3rd generation OCs, and their ability to counteract the effect of ethynyl oestradiol.

0514

RITUXIMAB IN THE TREATMENT OF ACUTE REFRACTORY AND CHRONIC RELAPSING THROMBOTIC THROMBOCYTOPENIC PURPURA: RESULTS FROM 28 PATIENTS.

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Background. Thrombotic thrombocytopenic purpura (TTP) is a life threatening disorder associated with ADAMTS 13 deficiency . Plasma exchange (PEX) remains the primary treatment modality in acute TTP, with various immunosuppressive agents (e.g. methylprednisolone (MP), vincristine, cyclosporine (CSA)) and other disease modifying agents (e.g. defibrotide), added in refractory and chronic relapsing cases. Mortality remains at 15-20%. *Methods.* We treated 28 patients; 26 were treated with Rituximab during an acute episode and 2 patients were treated electively as outpatients. 18 cases had acute refractory disease not responding to standard PEX and MP. A further 7 cases had previous acute TTP episodes, treated on relapse with Rituximab and PEX. An additional case was a 4 year old presenting with acute acquired TTP. She was successfully treated with BPL 8Y and Rituximab. Of the 2 cases treated electively, Rituximab therapy was dictated by ADAMTS 13 activity and IgG antibodies to ADAMTS 13, to avoid previous TTP associated complications. *Results.* 22 cases were female and 6 male. 75% had a history of neurological symptoms and 11% had cardiac TTP. Rituximab therapy consisted of 2-8 weekly doses of 375mg/m^2. All acute patients attained normal laboratory parameters and clinical remission within a median of 11 (range 0-33) days as defined by the number of PEX post the 1st Rituximab treatment. 26 patients had initial ADAMTS 13 activities <5% (normal range (NR) 66-126% by collagen binding assay) and 23 patients had normal ADAMTS 13 activity (median 78%) following Rituximab. IgG antibodies to ADAMTS 13 pre Rituximab were detected in 27 patients and were undetectable in all patients post Rituximab. The median number of PEX pre Rituximab was 13 (2-35) and following the first Rituximab treatment, 11 (0-33), including weaning with an alternate day regime. There was a significant reduction in the number of PEX's following the first dose of Rituximab (p=0.03 students paired t-test). To date, 6 patients required more than 4 Rituximab treatments as determined by ADAMTS 13 activity and IgG antibody to ADAMTS 13. Follow-up, 1-34 months, there have been 1 relapse in a patient lost to follow up. She was successfully re-treated with Rituximab. A further patient, treated with Rituximab 24 months previously, had a decrease in ADAMTS 13 activity to 10%. Elective retreatment with 4 weekly Rituximab resulted in normal ADAMTS 13 levels. Normalisation of CD 19 levels, between 6-15 months has not been associated with relapse. *Conclusion.* The data suggests patients with TTP respond promptly to Rituximab, with a significant reduction in the requirement for PEX. Our results suggest patients with TTP respond promptly to Rituximab and require significantly fewer PEX. In addition, Rituximab causes a reduction in IgG antibodies to ADAMTS 13 and appear to be strongly associated with disease remission. Rituximab therefore appears to be a safe, effective, targeted therapy for TTP.

Paroxysmal nocturnal hemoglobinuria and Fanconi Anemia

0515

A RANDOMIZED CONTROLLED PROSPECTIVE CLINICAL STUDY COMPARING THE COMBINATION THERAPY OF DEFERIPRONE (L1) AND DESFERRIOXAMINE WITH L1 AND DFO MONOTHERAPY IN PATIENTS WITH THALASSEMIA MAJOR

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This is the first randomized controlled prospective clinical trial comparing the combination therapy of deferiprone (L1) and desferrioxamine (DFO) with L1 and DFO monotherapy including changes of liver iron concentrations as an efficacy parameter during a period of one year in patients with thalassemia major. A total of 95 patients with thalassemia major were randomized into one of the following 3 treatment arms: L1 was given orally at a daily dose of 75 mg/kg either alone, in combination with s.c. DFO (40-50 mg/kg twice weekly) or DFO was given alone at a dose of 40-50 mg/kg 5 days a week (control arm). All patients had been treated with DFO prior to the study. Liver iron concentration (LIC) was measured by atomic absorption spectroscopy in biopsies at baseline and after one year. Biochemistry measurements including serum ferritin (SF) and liver enzymes were performed at 3-monthly intervals. Blood counts were analyzed weekly for 8 weeks and thereafter bi-weekly. Cardiac function (ECHO) was assessed 6-monthly. The average urinary iron excretion (UIE) of weeks 1, 12, 26, 38 and 54 was calculated. In patients receiving combination, UIE was measured on two different days, i.e. during L1 monotherapy and combination therapy. In total, fourteen patients (15%) dropped out from the study: one due to biopsy failure at baseline, five withdrawals from informed consent, four non-compliance to treatment, one due to jaundice, one died from arrhythmia induced heart failure in week 1, and two developed agranulocytosis at week 8 and 26, respectively. The average change in LIC after one year was most pronounced in the combination arm (-59%; p=0.0003), but LIC was also statistically significantly lowered after one year monotherapy with L1 (-35%; p=0.0003) or DFO (-51%; p=0.00004). The majority of patients in all treatment arms showed a clear decrease in SF and the mean SF was significantly reduced in the combination arm after one year (-2-120 microg/L; p=0.0003). The left ventricular ejection fraction increased during combination therapy (+3.4% absolute units; p=0.25), whereas it slightly decreased (-2.1% absolute units; p=0.41 and p=0.49, respectively) after either single agent treatment. The mean daily UIE was higher in L1-containing regimens than with DFO single agent therapy. UIE on days of combination (0.90±0.33 mg/kg/24h) was significantly higher than on days of L1 monotherapy (0.53±0.26 mg/kg/24h) (p=0.0003). The most common adverse events were transient increase in liver enzymes, nausea and arthralgia. The most serious adverse event was agranulocytosis observed in 2 patients (2.1%). This study indicates that the combination of daily L1 and twice weekly DFO at standard doses is an efficacious and safe chelation therapy for patients with thalassemia major.

0516

THE CHALLENGE OF FANCONI ANEMIA DIAGNOSIS IN PATIENTS WITH BONE MARROW FAILURE

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Background. Early diagnosis of Fanconi Anemia (FA) in patients with bone marrow failure (BMF) is crucial for optimal management, especially when deciding for lower-dose conditioning regimens in Haematopoietic Stem Cell Transplantation. Several FA diagnostic tests are available but, because of the remarkably high clinical variability and the potential emergence of revertant hematopoietic cells (somatic mosaicism), identifying patients with FA or ruling out this diagnosis is often challenging. *Aim.* To evaluate FA diagnosis in patients with BMF and no clear initial evidence of FA, using of a combination of classical and innovative tests in blood and fibroblasts. *Patients and Methods.* A cohort of 65 patients with BMF and no strong clinical evidence of FA was analysed. We initially classified patients in 3 groups: those likely to have idiopathic aplastic anemia (IAA) [n=31, group-1], those likely to have a constitutional condition other than FA (familial history and/or congenital abnormalities) [n=18, group-2], and

those likely to have IAA but who had isolated clinical positive findings which could also be present in FA [n=16, group-3]. Patients with a known or obvious FA diagnosis were not included in the study. Chromosome breakage test and FANCD2 immunoblot were performed in PBL in all patients [n=65]. Also, skin primary fibroblasts were analysed [n= 40] to detect potential hematopoietic FA reversion. Because chromosome breakage tests are barely efficient in fibroblasts, we performed FANCD2 immunoblot and developed a new flow cytometry test based on MMC-sensitivity in fibroblasts to identify FA/BRCA *downstream* groups. *Results.* In total, 4 patients with FA were identified. The only positive clinical findings for those patients were: Patient-1, group-3 (precocious menopause, vocal cord neoplasia at age 38yo), Patient-2, group-3 (BMF at age 10yo following a period of isolated thrombocytopenia, 1 café-au-lait spot, hypospadias), Patient-3, group-2 (low birth weight/short stature, 'peculiar' facies, onset of pancytopenia only at age 35yo) and Patient-4, group-1 (10yo, no positive clinical findings; did resemble IAA). The two patients from groups 1 and 2 were diagnosed with chromosomal breakage test, and further classified with FANCD2 immunoblot in PBL. For the two patients from group-3, based on persistent clinical suspicion of FA after negative breakage test in PBL, somatic mosaicism with complete haematopoietic reversion was diagnosed using FANCD2 immunoblot fibroblast analysis. Importantly, FA diagnosis was definitely excluded in all other patients. *Conclusions. In situ* ations where the suspicion of FA persists after a negative breakage test in PBL (e.g.: congenital physical abnormalities and possible mosaicism), then diagnostic tools should be performed on fibroblasts. As a rule, we found that underdiagnosing FA is very rare if careful history and physical exam are done together with standard chromosome breakage tests in PBL. Because no cases of FA were found in our cohort of patients with IAA presentation and negative breakage test, we suggest that screening can be limited to this technique. The strategy here presented allowed us to identify a few unexpected FA cases in a cohort of BMF patients, and importantly, to definitely rule out FA in others.

0517

THE EFFECT OF COMBINED THERAPY WITH DEFEROXAMINE AND DEFERIPRONE ON MYOCARDIAL IRON AND ENDOTHELIAL FUNCTION IN THALASSEMIA MAJOR: A RANDOMIZED CONTROLLED TRIAL USING CARDIOVASCULAR MAGNETIC RESONANCE

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Background. In β-thalassemia major (TM) cardiac failure secondary to myocardial iron loading remains the leading cause of death. Approximately two-thirds of patients maintained on deferoxamine continue to exhibit myocardial iron loading. The oral iron chelator, deferiprone has been demonstrated to remove myocardial iron and it has been proposed that in combination with deferoxamine it may have an additive effect. Myocardial iron can be rapidly and reproducibly quantified using a cardiac magnetic resonance (CMR) T2* technique. Endothelial function (as quantified by flow mediated dilatation of the brachial artery (FMD)) can be impaired in TM which may further contribute to cardiovascular pathology. FMD is also reliably measured by CMR. CMR is therefore well suited to assess the efficacy of new therapies for the treatment of iron overload in TM. *Aims.* To report the changes in myocardial iron loading (changes in T2*) and endothelial function (as assessed by FMD) from a randomized placebo controlled trial comparing the combined therapy of deferiprone and deferoxamine with the standard therapy of deferoxamine alone. *Methods.* A mobile CMR scanner (1.5T, Siemens Sonata) was transported to Cagliari, Italy. The myocardial T2* was assessed in 167 patients with TM. 65 patients (male 27, female 38, age 29±4.8years) with mild-moderate myocardial iron loading (T2* 8-20ms) were randomized to receive either deferoxamine and placebo, or deferoxamine and deferiprone. Myocardial and hepatic T2* were assessed at baseline, 6 and 12 months. Endothelial function was assessed at 0 and 12 months. *Results.* Analysis of covariance showed a significant difference between the two groups, with the combined group showing superior effects in reducing both myocardial iron (p=0.017) and hepatic iron (p<0.001). See figure 1. Over the 12 months endothelial function improved significantly in the combined treatment group (from 10.5% to 18.3%, p<0.001) but not in the placebo controlled group (9.9% to 13.4%, p=0.10). *Conclusion.* In patients with mild-moderate cardiac iron loading the combined therapy of deferiprone and deferoxamine is superior to deferoxamine alone in the removal of myocardial iron and improving endothelial function.

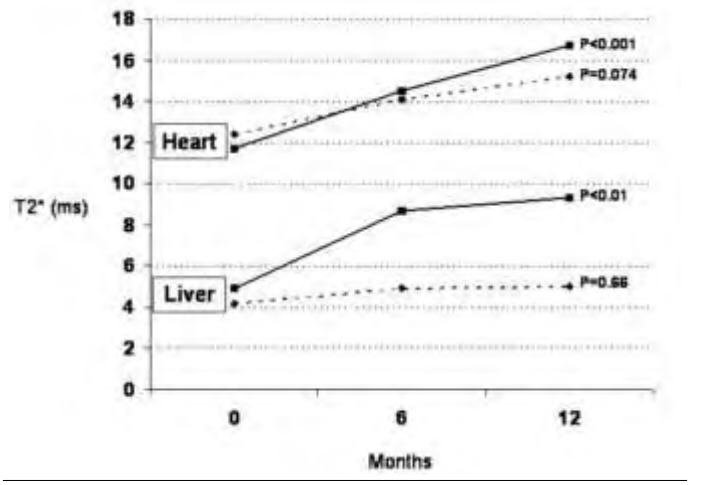

Figure 1. Both heart and liver iron loading significantly improve with combined chelation therapy (*continuos line*). There is no significant improvement in the placebo group (*dashed line*).

0518

PAROXYSMAL NOCTURNAL HAEMOGLOBINURIA: LONG-TERM EPIDEMIOLOGICAL STUDY

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Background. Paroxysmal nocturnal haemoglobinuria (PNH) is a rare acquired disorder of haematopoietic stem cells. Although knowledge about the pathophysiology of the disease is increasing, few studies have been published on the long-term follow up mainly because of the rarity of the disease. *Aims.* Analyzing a large cohort of patients with PNH on the long term to better determine prognostic factors. Assessing the role, if any, of the introduction of flow cytometry for diagnosis in the presentation and following of the disease. *Methods.* We have already reported such an analysis on 220 patients in 1996 (Socié *et al.,* Lancet). Data were updated and collected on an additional 258 patients with PNH. Haematological centres were contacted by the way of the French Society of Haematology and/or the French Association of Young Haematologist. The date of diagnosis was based on blood cytometric analysis if there was no prior positive Ham's test. Data validation is still in progress. *Results.* Provisory results are the following. We report here the natural history of PNH among 478 patients (258 female, 220 male). 58 French haematological centers participated in the study. Patients were diagnosed over a 55-year period (1950-2005). The age at diagnosis was 34 (inter-quartile range: 24-47). The median follow up (± standard deviation) is 5.6 years (±0.4). 50 patients underwent allogeneic bone marrow transplantation. During the evolution, 113 patients presented a thrombosis, 9 a myelodysplasia, and 6 an acute leukemia, respectively. Ninety six patients died. The Kaplan-Meier survival (± standard deviation) was 85% at 5 years (± 2), 76% at 10 years (± 3), and 66% at 15 years (± 3). The analysis of prognostic factors are on going at time of abstract submission. *Conclusion.* This is the largest cohort of patients with PNH reported until now. Definitive results after complete validation of the data base will be presented at the meeting.

0519

HIGH PREVALENCE OF PULMONARY HYPERTENSION AND HEMOLYSIS ASSOCIATED NITRIC OXIDE DEPLETION IN PATIENTS WITH PAROXYSMAL NOCTURNAL HEMOGLOBINURIA

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Background. Pulmonary hypertension (PHT) is an emerging common complication of hereditary hemolytic anemias. It has been mechanistically and epidemiologically linked to intravascular hemolysis and decreased nitric oxide (NO) bioavailability. The release of excessive red cell hemoglobin during intravascular hemolysis can exceed the capacity of the hemoglobin scavenging molecule, haptoglobin, leading to the consumption of endogenous NO. While this complication has been described in approximately 30% of adult patients with sickle cell disease and thalassemia, the prevalence of PHT in patients with paroxysmal nocturnal hemoglobinuria (PNH), an acquired disease with the highest levels of intravascular hemolysis observed, has never been determined. PNH patients frequently have symptoms consistent with both hemolysis and PHT including severe fatigue and dyspnea on exertion. *Aims.* We, therefore, examined for the presence of PHT in PNH and explored potential mechanisms associated with its development by measuring the ability of plasma to instantaneously consume NO. *Methods.* Doppler echocardiography was performed in 28 hemolytic PNH patients to estimate pulmonary artery pressures. Transmitral flow, Doppler determinations of the severity of valvular regurgitation, and left ventricular stroke volume were assessed and graded. Systolic PHT was prospectively defined by a tricuspid regurgitant jet velocity (TRV) of at least 2.5m/s at rest. Nitric oxide consumption was assessed using ozone-based chemiluminescence, and red cell hemolysis was determined by plasma levels of lactate dehydrogenase (LDH). Blood was collected using methodologies to limit artefactual hemolysis. *Results.* Tricuspid regurgitation was observed in 20 out of 28 patients with PNH. Fourteen of these 20 evaluable patients (70%) demonstrated elevated pulmonary artery systolic pressures. Twelve (60%) had mild to moderate PHT (mean TRV 2.6m/s±0.1) while two (10%) had moderate to severe pressures (mean TRV 3.7m/s±0.2). Plasma from PNH patients (n=32) consumed 34.6±8.3 micromolar NO while normal subjects (n=9) consumed 2.2±0.6 micromolar NO ($p<0.0001$). LDH levels correlated with NO consumption (r=0.6342, $p=0.0002$). Eculizumab is a humanized monoclonal antibody that binds to C5 inhibiting terminal complement activation. In a separate cohort of 7 patients treated with eculizumab for a median of 3 years to reduce hemolysis, the ability to consume NO appeared lower (13.2±4.8 micromolar NO). *Conclusions.* 1) PHT has a much higher prevalence in PNH than in other hemolytic disorders. 2) Patients with PNH demonstrate high levels of NO consumption that are highly correlated to intravascular hemolysis (LDH) in these patients. 3) Eculizumab therapy is associated with reduced levels of NO consumption. Additional studies are required to determine the contributions of intravascular hemolysis and reduced NO bioavailability to the pathogenesis of pulmonary hypertension in PNH and the possible role of PHT in the morbidity and mortality characteristic of the disease.

Cell signaling, transcriptional control and apoptosis - I

0520

ONTOGENY, FUNCTION AND PERIPHERAL HOMEOSTASIS OF REGULATORY T CELLS IN THE ABSENCE OF INTERLEUKIN-7

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Background. Mice lacking Interleukin 7 (IL-7-/- mice) are lymphopenic, but show no signs of autoimmunity, contrary to what is observed in other lymphopenic models. *Aims.* We investigated whether the absence of disease was due to the fact that IL-7 is dispensable for the ontogeny, function and homeostasis of regulatory Foxp3-expressing CD4+ T cells. Alternatively, the absence of IL-7 might directly prevent manifestations of T-cell mediated disease. *Methods.* Frequencies of CD4+CD25+ T cells in the thymus and spleen of IL-7-/- mice were assessed by Flow Cytometric analysis. We studied the expression of Foxp3 in thymic and splenic CD4+CD25+ T cells from IL-7-/- mice. The potential of pathogenic and regulatory IL-7-/- T-cells was evaluated in co-transfer experiments. Finally, CD25+CD4+ T cells in IL-7-/- and control mice were depleted by 10 daily injections of PC61mAb (anti-CD25), and microscopic analysis of the colon was subsequently carried out. *Results.* We show here that the establishment of the peripheral pool of FoxP3-expressing regulatory CD4+CD25+ T cells is IL-7 independent, and the premature involution of the thymus in IL-7-/- mice does not change the representation of CD4+CD25+ T cell compartment. The frequency of peripheral activated CD4+ T cells increases with age in both CD25- and CD25+ compartments, with CD4+CD25+ T cells displaying signs of constant activation. IL-7-/- CD4+CD25+ T cells control Inflammatory Bowel Disease induced by IL-7-/- CD25-CD45RBhigh T cells, even in hosts lacking IL-7. Depletion of CD25+ T cell subset after thymic involution results in a mild form of IBD, which resolves concomitantly with the regeneration of this subset. *Conclusion.* This study shows, for the first time, that IL-7-/- mice have a robust regulatory FoxP3-expressing CD4+ T cell compartment that controls T-cell mediated disease. It also highlights the potential of regulatory FoxP3-expressing CD4+CD25- T cell population to restore a functional CD4+CD25+ T cell compartment through an IL-7 independent pathway.

0521

LYSOSOMAL ROUTING OF G-CSF RECEPTORS DEPENDS ON A SINGLE MEMBRANE-PROXIMAL LYSINE RESIDUE, IS CONTROLLED BY SOCS3 AND PLAYS A CRITICAL ROLE IN G-CSF-INDUCED GRANULOPOIESIS

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Background. The G-CSF receptor (G-CSF-R) tightly controls proliferation, survival and differentiation of myeloid progenitor cells. Mutations truncating the C-terminus of G-CSF-R are found in severe congenital neutropenia (SCN) patients at risk to develop AML. Myeloid progenitor cells expressing truncated G-CSF-R hyperproliferate in response to G-CSF and show defective differentiation. These truncated G-CSF-R have lost their recruitment site for SOCS3 and are hampered in receptor internalization. *Aims.* To study the role of receptor internalization and postendocytic routing in the control of G-CSF signaling and to determine the underlying mechanisms of these processes. *Methods.* We studied internalization rate, post-endocytic trafficking, signal transduction and proliferation/differentiation characteristics of various mutant G-CSF-R. Routing was monitored by confocal microscopy following double labeling of internalized anti-G-CSF-R antibodies and endosomal marker proteins EEA1 (early endosomes), Hrs (prelysosomal endosomes), Rab7 (late endosomes/lysosomes) and Lamp1 (lysosomes). Single amino acid mutants of G-CSF-R were generated to study involvement of individual lysines and the role of SOCS3 in receptor routing. Signaling activity was assayed in primary cell cultures and 32D cells and in STAT5 reporter and band shift assays. *Results.* We show that internalized G-CSF-R follow the classical endosomal-lysosomal degradation route. A lysine-null (K5R) G-CSF-R was retained in an Hrs-positive prelysosomal compartment and not targeted for degradation, indicating that lysosomal routing

depended on one or more lysines in the cytoplasmic tail of the G-CSF-R. Importantly, 32D cells expressing G-CSF-R/K5R displayed strongly prolonged STAT5 activity, increased proliferation and defective granulocytic differentiation, indicating a pivotal role for lysine-mediated receptor routing in G-CSF signal attenuation. Similar results were obtained in colony assays with G-CSF-R deficient primary bone marrow progenitors transduced with K5R or wt G-CSF-R. Using single K to R G-CSF-R substitution and add-back mutants, we subsequently established that lysosomal routing, downregulation of STAT5 activity and induction of myeloid differentiation all depend on the integrity of a single, membrane-proximal lysine residue of G-CSF-R, i.e., K632. Only the add-back of K632 but not of any of the other lysines to the lysine-null G-CSF-R fully restored routing as well as proliferation/differentiation signaling characteristics of G-CSF-R. The suppressor of cytokine signaling-3 (SOCS-3) is a prominent negative regulator of G-CSF-induced STAT5 activity and recruits E3-ubiquitin ligase activity via its SOCS-box. We found that SOCS-3-mediated inhibition of G-CSF-induced STAT5 activation strongly depended on the presence of the SOCS3 SOCS-box as well as on G-CSF-R K632. In addition, lysosomal routing of mutant G-CSF-R/Y729F, lacking the SOCS-3 binding site, was severely diminished. *Summary/conclusions*. Receptor internalization and routing are important mechanisms by which proliferation signals from G-CSF-R are attenuated and neutrophilic differentiation is induced. The presence of a single lysine residue (K632R) and the SOCS-3 recruitment site (Y729) in G-CSF-R are both critical for the control of lysosomal routing and attenuation of G-CSF-induced proliferation. We thus present evidence that SOCS-3 is a major regulator of G-CSF-R routing, most likely via recruitment of E3-ubiquitin ligase for G-CSF-R/K632, thereby playing a critical role in maintaining the proliferation/differentiation balance during G-CSF-activated neutrophil production.

0522

TRANSLATION OF IGBP1 MRNA CONTRIBUTES TO THE REGULATION OF EXPANSION AND DIFFERENTIATION OF ERYTHROID PROGENITORS

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Background. Erythroid progenitors can be expanded *in vitro* in the presence of erythropoietin (Epo) and stem cell factor (SCF), while they differentiate to enucleated erythrocytes in presence of Epo only. Previously we showed that SCF-induced delay of differentiation depends on the activation of phosphoinositide-3-kinase (PI3K). An important PI3K-dependent process in cell proliferation is regulation of mRNA translation. PI3K controls the activity of mTOR (mammalian target of rapamycin), whose activation results in phosphorylation of eIF4E-binding protein (4E-BP). Fully phosphorylated 4E-BP releases eIF4E (eukaryote Initiation Factor 4E), which can subsequently bind eIF4G, the scaffolding protein of the eIF4F cap-binding complex. In particular mRNAs with a structured UTR (untranslated region) require optimal availability of eIF4E to be translated. SCF, but not Epo can induce full phosphorylation of 4E-BP and efficient formation of the eIF4F cap-binding complex. Overexpression of eIF4E inhibited erythroid differentiation, indicating that SCF-induced formation of the eIF4F complex contributes to progenitor expansion. A major step in mRNA translation controlled by eIF4F is polysome recruitment. *Aims.* Our objective is to identify genes that are translationally controlled upon SCF signalling and to investigate their contribution in the attenuation of erythroid differentiation. *Methods.* To identify genes whose expression is regulated by signaling-induced polysome recruitment, we compared total and polysome-bound mRNA from factor deprived and Epo plus SCF restimulated progenitors on gene-expression micro-arrays. Profiling was performed with 4 biological replicates and candidate genes were selected using ANOVA. In subsequent cluster analysis we combined these data with (polysomal) expression profiles of differentiating erythroid cells. Real time PCR was used to investigate if polysome recruitment of candidate transcripts are dependent on PI3K activation and eIF4E expression. Retroviral transduction was used to constitutively express these genes in the erythroid cell model and cell number, cell size and hemoglobinisation was measured to assess the effect on erythroid differentiation. Western blot analysis was used to investigate the effect of constitutive active IGBP1 on the phosphorylation state of mTOR targets. *Results.* We identified genes, upregulated specifically at the level of mRNA polysome recruitment and downregulated during erythroid differentiation. We further characterised 13 genes whose polysome recruitment is dependent on the PI3K/mTOR pathway. Constitutive expression of these targets in erythroid progenitors revealed that IGBP1 (Immunoglobulin binding protein 1) was able to inhibit erythroid differentiation. We elucidated a mechanism by which the IGBP1/PP2A complex prolongs the phosphorylation of mTOR targets, possibly the mechanism inhibiting erythroid progenitor differentiation. *Summary/Conclusions.* We identified in this study a novel and unique set of genes that are minimally regulated at the level of transcription and are recruited to polysomes upon SCF signalling. We support the importance of translation control to regulate the balance between expansion and differentiation of erythroid progenitors, by showing that IGBP1, a translationally controlled gene, blocks erythroid differentiation and this can be explained by maintenance of mTOR target phosphorylation under differentiation conditions. This suggests that, like SCF signalling and eIF4E overxpression, IGBP1 enhance translation initiation efficiency.

0523

EXPRESSION OF THE TEL-AML1 LEUKEMIA-ASSOCIATED FUSION PROTEIN INHIBITS TGF-BETA1 SIGNALING

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Background. The TEL-AML gene fusion is the most frequent chromosome translocation generated abnormality in childhood leukemia; ~20% of acute lymphoblastic leukemia (ALL). The translocation usually arises before birth probably as an initiating event, and its impact is to generate a clone of covert, clinically silent pre-leukemic B cell progenitors. ALL disease arises following a second, post-natal hit/genetic event occurring up to 14 years later, usually involving deletion of the normal TEL allele. The mechanism by which TEL-AML1 protein transforms and sustains early progenitor B cells is currently unknown. The chimeric gene encodes a protein in which TEL dimerization domains are fused N-terminally to all the DNA binding and activating domains of AML1 and there is experimental data to endorse the probability that the activity of the TEL domain converts AML1 to a transcriptional repressor, probably via the recruitment of co-repressor molecules to the transcriptional complex. *Aims.* To investigate how TEL-AML1 expression provides the pre-leukemic clone with selective advantage. In particular, considering the critical role of TGF-β1 in the growth and differentiation of hematopoietic cells, to test whether TEL-AML1 interferes with TGF-β1 signaling. *Methods.* We performed this study using a TEL-AML1 inducible expression system. We have established cell lines of murine Ba/F3 pro-B cells that stably express a regulatory, truncated human progesterone receptor ligand-binding domain (pSwitch, Invitrogen) that undergoes a conformational change in the presence of the steroid agonist, mifepristone and conversion to an active form. The active binding domain is then able to bind and activate transcription from an otherwise silent TEL-AML1 gene that we have also stably introduced into these cells. *Results.* Ba/F3 cells expressing TEL-AML1 grow more slowly than controls but are more resistant to the anti-proliferative effects of TGF-β1 (at 10 ng/mL). Using transient transduction reporter assays with a construct that contains the mouse germline promoter Ig-α, positively regulated by TGF-β1, we have shown that expression of TEL-AML1 markedly represses the response of the promoter to TGF-β1. The transcriptional response to TGF-β1 is Smad 2/3 dependent and the Runt domain of AML1 is known to bind Smad3. We confirmed that TEL-AML1 (which includes the Runt domain) associates with Smad3 by immunoprecipitation. *Conclusions.* On the basis of these observations, we propose that while AML1 cooperates with Smad proteins to induce transcription in response to TGF-β1, the TEL-AML1 fusion encoded protein interferes negatively with the TGF-β1 signaling. We speculate that the fusion protein, in virtue of its association with Smad3, may decrease the TGF-β1 transcriptional activity by sequestering Smads away from their co-operators and/or by recruiting co-repressor molecules to the transcriptional complex at the Smad consensus DNA domains. Since TGF-β1 is a critical regulator of cell growth, cellular senescence, differentiation and apoptosis, the alteration of its signaling activity may play an important role in the establishment of the persistent pre-leukemic clones by TEL-AML1 and/or in their progression to overt leukemia.

0524

ONCOGENIC CBL MUTANTS CONFER A TRANSFORMING POTENTIAL TO HEMATOPOIETIC CELLS EXPRESSING THE FLT3 TYROSINE KINASE

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Background. CBL is a E3-ubiquitin ligase, that negatively regulates many receptor tyrosine kinases (RTK). The CBL gene is located on the human chromosome 11q23. Balanced translocations (t(4;11); t(11;14) and interstitial deletions involving CBL and a CBL-MLL fusiongene were described in patients with B-cell lymphomas and acute leukemias. Two oncogenic CBL deletion mutants, CBL-70Z and v-CBL, were isolated from murine retroviruses inducing B-cell lymphomas and acute leukemias in mice. The Fms-like tyrosine kinase 3 (FLT3) is expressed by the leukemic cells of 70-90% of patients with acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL) and can contribute to malignant transformation by harbouring activating mutations, aberrant autocrine stimulation or overexpression. *Aim*. We hypothesised that CBL could play a role in regulating FLT3 RTK activity and, if mutated, could lead to aberrant FLT3 signalling in acute leukemias. *Methods.* CBL-WT and the CBL mutants (v-CBL, CBL70Z) were stably expressed alone or with FLT3-WT (FLT3-WTCBL-WT, FLT3-WTCBL-70Z and FLT3-WTv-CBL cells) in murine IL-3 dependent Ba/F3 cells. Proliferation assays in the absence of IL-3, presence of FLT3 ligand (FL) and selective FLT3 inhibitors were performed. FLT3 activation status und the activation of downstream signaling pathways were investigated by western blotting. *Results.* Stable coexpression of FLT3-WT and CBL-70Z or v-CBL, but not FLT3-WT with CBL-WT or one construct alone, conferred IL-3 independent growth to Ba/F3 cells. Stimulation with FLT3 ligand (FL) lead to hyerproliferation of FLT3-WTCBL-70Z and FLT3-WTv-CBL expressing cells, but not of FLT3-WTCBL-WT expressing cells. To determine whether the proliferative advantage of FLT3-WTCBL-70Z and FLT3-WTv-CBL cells is mediated by FLT3 we cultivated the cells in presence of selective FLT3 inhibitors, SU5614 and PKC412. Both inhibitors were able to abrogate the IL-3 independent and FL-stimulated growth of FLT3-WTCBL-70Z and FLT3-WTv-CBL cells. The FLT3-WT receptors were constitutively activated and showed a higher spontaneous dimerization rate in FLT3-WTCBL-70Z and FLT3-WTv-CBL expressing cells in the absence of FL. Analysis of the three important downstream signaling pathways of FLT3 (STAT5-, PI3K/AKT- and MAPK-pathway) could show activation of STAT5 and AKT in FLT3-WTCBL-70Z and FLT3-WTv-CBL expressing cells. *Summary.* The CBL deletion mutants, CBL-70Z and v-CBL, but not CBL-WT, confer a transforming potential to hematopoietic cells expressing FLT3. The pro-proliferative phenotype of FLT3-WTCBL-70Z and FLT3-WTv-CBL cells is mediated by an increase in FLT3 tyrosine kinase activity compared to FLT3-WTCBL-WT cells and can be inhibited by selective FLT3 inhibitors. Here, we provide a new mechanism of transformation mediated by FLT3: mutations of a regulatory protein, that is implicated in negative regulation of RTK kinase activity.

Multiple Myeloma - Clinical / Experimental

0525

MYELOABLATIVE ALLOGENEIC STEM CELL TRANSPLANTATION AS PART OF FIRST-LINE THERAPY FOR *DE NOVO* MULTIPLE MYELOMA : LONG-TERM FOLLOW-UP OF PATIENTS REGISTERED IN THE FRENCH SOCIETY OF STEM CELL TRANSPLANTATION DATABASE (SFGM-TC).

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Background. The role of myeloablative allogeneic stem cell transplantation (alloSCT) in the treatment of multiple myeloma (MM) is still an area of controversy. Few series have described the results of such a therapy as part of first-line treatment in younger patients. *Methods.* From 1985 to 2003, 116 patients with *de novo* MM less than 55 years were treated by alloSCT within 12 months following diagnosis either in first CR (n = 13), first PR (n = 70), stable disease (n = 14) or primary refractory (n = 19), and registered in the SFGM-TC database. *Results.* Patients were 67 males, 49 females, median age 41 years (18-53). The donor was a geno-identical one in 105 cases, and pheno-identical in 11 cases. The conditioning regimen consisted of 12 Gy total body irradiation (TBI) + 120 mg/kg Cyclophosphamide in 50% of the cases, TBI + Cy + melphalan in 20% of the cases, and TBI + melphalan in 17% of the cases. T-cell depletion was used in only 8 cases, and GVHD prophylaxis consisted of the combination of cyclosporine A + short course methotrexate in 85% of the cases. Grade 3-4 acute GVHD was documented in 18.7% and chronic GVHD in 30.5% of the cases, respectively. 100 days after alloSCT, the mortality rate was 23%, and 40% 1 year after alloSCT. The overall survival was 41% at 4 years, and 31% at 12 years, and disease-free survival was 26% at 12 years with a true plateau observed 5 years after alloSCT. Chemosensitive disease at the time of alloSCT and occurrence of chronic GVHD were associated with a better survival (12-year survival 38.2 vs 18.2% p=.02, and 42 vs 20.1% p=.002, respectively). *Conclusion.* With a prolonged follow-up, data from the SFGM-TC show that alloSCT when performed as part of first-line therapy, despite a high initial mortality, may cure one quarter of the patients with *de novo* MM less than 55 years.

0526

NEW CRITERIA TO IDENTIFY RISK OF PROGRESSION IN SMOLDERING MULTIPLE MYELOMA: MULTIPARAMETRIC FLOW CYTOMETRY ANALYSIS OF BONE MARROW PLASMA CELLS

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Background. Smoldering multiple myeloma (SMM) is a monoclonal disorder defined by the presence of a serum monoclonal protein ≥ 3g/dL or bone marrow plasma cells ≥10% and absence of end-organ damage. These patients require close follow-up because the high risk of progression to symptomatic multiple myeloma. Therefore, the definition of new parameters that could be used for the identification of patients at the highest risk of progression could be of great importance in the clinical setting. *Aims.* To evaluate the impact of immunophenotypical analysis of bone marrow (BM) plasma cell (PC) for the prediction of risk of progression of SMM. *Methods.* From January 1996 to September 2004, bone marrow aspirate samples from 78 patients, who fulfil the criteria of SMM according to the International Myeloma Working Group criteria, were analysed by multiparametric flow cytometry. Plasma cells were immunophenotypically classified as normal or abnormal according to the expression of CD38, CD45, CD19 and CD56 antigens. Other parameters included were: percentage of plasma cell infiltration by morphology and cytometry, MC, immunoparesis, presence of Bence-Jones pro-

teinuria, haemoglobin, platelets, calcium, and albumin. The median age of the series was 69 years (range 43-88). The monoclonal paraprotein was IgG in 55 (65%), and IgA in 29 (34%), with a median paraprotein level of 2.5 mg/dL. The median follow-up was 50 months. *Results.* Thirty six patients (42%) progressed to MM, with a median time to progression (TTP) of 26 months (range 2 to 94 months). Interestingly, flow cytometry showed that the number of aberrant PC (aPC) within the total PC (TPC) population in BM, clearly predict the risk of progression. Thus, in patients with ≥ 95% aPC from TPC the median TTP was 40 months vs not reached in the rest (p=0,0000). Other parameters with a significant influence on progression in the univariate analysis were: the paraprotein level (higher vs lower 3 mg/dL. p= 0.0017), the presence of immunoparesis (no paresis vs. one or two Ig. p= 0.0001), the presence of Bence-Jones proteinuria (p=0.017), the total BM infiltration assessed both by morphology and flow cytometry (p=0.0324; and p=0.0001, respectively). Moreover, this cut off level of 95% aPC within TPC, also allow us to discriminate two risk categories upon considering only patients at low riesk of progression, based on a low paraprotein level or absence of inmunoparesis (p=0,0072 and p= 0,0056, respectively). By multivariate analysis this new parameter (95% aPC from TPC), together with immunoparesis (no vs one or two Ig), BM infiltration by cytometry, and the amount of MC, had independent significant impact on risk of progression. *Conclusion.* bone marrow immunophenotypical analysis of plasma cells by multiparametric flow cytometry at diagnosis is useful for predicting progression of SMM into active MM.

0527

A MULTICENTER, RANDOMIZED TRIAL OF ZOLEDRONATE VS OBSERVATION IN EARLY-STAGE, ASYMPTOMATIC MYELOMA

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Background. The use of bisphosphonates in patients with asymptomatic, otherwise untreated early-stage myeloma is still debated and currently not recommended by evidence-based guidelines due to a substantial lack of appropriate studies. Zoledronate is a third generation bisphosphonate, which significantly decreases skeletal events in active myeloma and has been demonstrated to have a possible anti-myeloma *in vitro* and *in vivo* effect. *Aims.* The aim of this study was to evaluate whether the *prophylactic* use of zoledronate is able to reduce the rate of and the time to evolution in overt, symptomatic myeloma requiring chemo-radiotherapy in this population of patients. *Methods.* On June, 2001, ten italian centers started a randomized clinical trial comparing administration of zoledronate vs simple observation in patients with monoclonal gammopathy fulfilling the diagnostic criteria of asymptomatic, stage IA, IIA or *smouldering* myeloma, not requiring chemo-radiotherapy. Patients strictly diagnosed as having *true* MGUS were excluded. One-houndred-sixty patients were enrolled and randomized (1:1) to receive (n. 80) or not (n. 80) zoledronate (Zometa, Novartis Pharmaceuticals, Origgio, Italy) for one year, on an out-patient basis, at the dose of 4 mg as 15' i.v. single monthly infusion. *Results.* No severe adverse events were recorded throughout the study, with the exception of a single patient treated with zoledronate who developed a reversible picture of osteonecrosis of the jaw.. In the observational arm. six patients were lost at follow-up after 6-20 months. Asymptomatic hypocalcemia, without need of interrupting the treatment and promptly corrected by substitutive therapy, occurred in fifteen of zoledronate-treated patiens. Fever developed in seven patients receiving zoledronate, one of whom stopped the treatment after 3 infusions. Overall, no significant reduction of M-component (> 25%) was observed. On intention-to-treat analysis, after a median follow-up of 42 months, there were 19 (23.7%) progressions requiring treatment in the zoledronate group and 24 (30%) within the controls (p n.s.). Median time-to-progression was 19 and 16 months, respectively (p n.s.). Among the 36 patients who required chemo-radiotherapy in both arms, bone lesions and/or hypercalcemia at the time of progression were observed in 16/20 (80%) of controls, and in 7/16 (43.7%) of zoledronate-treated patients (p<0.001). *Conclusions*. Our data indicate that the use of zoledronate in patients with early-stage, asymptomatic myeloma reduce the possibility of developing skeletal events at progression. Although a weak trend in favour of zoledronate arm was observed, in this study there was no statistical evidence about the possibility that zoledronate may also decrease the number of and/or the time to progression of the disease.

0528

ARRAY-CGH DETECTS FREQUENT RECURRENT IMBALANCES IN PLASMA CELL DISORDERS

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Background and *Aims.* Genomic imbalances such as losses and gains occur frequently in hematological cancers. Their characterization in plasma cell disorders (PCD) is largely incomplete and few lesions (mostly identified by conventional cytogenetics and FISH) have been extensively studied for their pathogenetic and prognostic role. There is thus a clear need for a genome-wide screening of cytogenetically-cryptic lesions, through sensitive, robust and reproducible approaches. Array-CGH allows obtaining a comprehensive view of genomic imbalances, with a precise mapping of these aberrations to the genomic sequence. It has proven extremely successful in several diseases including chronic lymphocytic leukemia (CLL) which closely resemble multiple myeloma (MM) in terms of clinical and molecular heterogeneity. We have screened a panel of patients with PCD with the following *Aims.* 1) to identify the most common yet undescribed genetic lesions; 2) to confirm these lesions by FISH; 3) to link genomic imbalances and clinical outcome. *Methods.* CD-138 purified plasma cells were employed. Genomic DNAs, from both the tumor and normal reference cells, labeled with different fluorescent dyes were cohybridized to 1 Mb resolution arrays (Spectral Genomics Inc. USA) containing 2600 Bacteria Artificial Chromosome (BAC) clones, according to manufacturers recommendations. Variations in DNA sequence copy number for each BAC clone was assessed by relative fluorescence signal intensities, providing a locus-by-locus measure of DNA copy-number changes. FISH experiments have been performed to confirm clonal abnormalities (gains/ losses) identified by array-CGH. BAC clones were labeled for FISH experiments and interphase nuclei were made, according to standard cytogenetic protocols. *Results.* 20 patients were studied including 16 MM and 4 PCL. The median number of lesions/patient observed in our panel was 17 (range 4-135). The amount of the genome affected by chromosomal imbalances was highly variable among different patients (median 3.9% range: 0.14%-27%). This number is superior to that reported in CLL (Drandi, ASH 2003) and to a lesser extent to diffuse large B cell lymphoma (DLBCL) (Chaganti, Blood 2005). Of 2600 BACs 934 were always spared from genetic damage, 864 were targeted only in one patient, 401 in two patients, 296 in 3-5 patients and 105 were targeted in six patients or more. By restricting the analysis to the most common lesions we have identified five previously undescribed recurrent lesions occurring in at least 30% of patients, involving the following regions: 19p13.2, 14q12, 16q12.1 11q24, 9q23. We have confirmed the first two lesions by FISH, while for the others experiments are ongoing. *Conclusions.* 1) In PCD the genome undergoes a high degree of genetic disruption compared to other lymphoid tumors, particularly CLL; 2) a number of highly recurrent lesions have been identified, and some have already been confirmed by FISH. All these lesions will require further investigation to identify candidate target genes and to verify if they might be prognostically relevant.

0529

HYPERDIPLOIDY AND HYPODIPLOIDY IN MONOCLONAL GAMMOPATHY OF UNDETERMINED SIGNIFICANCE AND MULTIPLE MYELOMA, AND THEIR RELATIONSHIPS WITH MONOSOMY 13

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Background. Cytogenetic performed in multiple myeloma (MM) allow the definition of two pathways for malignant progression, one hyperdiploid and the other hypodiploid. Monoclonal gammopathy of undetermined significance (MGUS) is probably a preliminary step to MM for some patients at least. Cytogenetic status in MGUS is limited to interphase FISH techniques, due to the small amount of abnormal plasma cells and to their low proliferation rate. *Aims.* To define incidence of hyperdiploidy and hypodiploidy in MM and MGUS, and the distribution of monosomy 13 within each group. *Methods.* We ascertained DNA content of plasma cells (ploidy) using Feulgen staining and image analysis in 96 MGUS and 169 MM patients. Interphase FISH was perfomed using centromeric probes to look for trisomies 3, 7, 9, 11 and 15 in 42 MGUS and using rb-1 gene probe for monosomy 13 in 57 MGUS and 150 MM patients. *Results.* Hyperdiploidy and hypodiploidy were found in 54% and 11.5%, and in 59.5% and 25% of MGUS and MM patients respectively. Mean and median ploidies, for hyperdipoid as for hypodiploid patients, were similar in MGUS and MM. Interphase FISH confirmed the association between trisomies for several odd chromosomes and hyperdiploidy. Monosomy 13 was found in 24.6% in MGUS and in 45.3% in MM: incidence was similar in hyperdiploid MGUS and hyperdiploid MM (38% and 31.9% respectively), whereas it was found in 11.1% of hypodiploid MGUS contrasting with 76.3% found in hypodiploid MM. Only two patients, both hyperdiploid, evolved to MM after a mean follow-up of 77 months. *Conclusions.* Our results show that the number of hyperdiploid patients and the amount of chromosomes gained are similar in MGUS and in MM; monosomy 13 was found in equal numbers in both disorders, hypothesizing that events unrelated to monosomy 13 need to occur for evolution of MGUS to MM. In contrast, hypodiploidy is rare in MGUS, and is unrelated to monosomy 13, hypothesizing that hypodiploid MM might occur either after a MGUS step with deletion 13 as a secondary event, or using another pathway without prior MGUS.

Presidential symposium: 6 best abstracts

0530

HSV-TK DONOR LYMPHOCYTES ADD-BACKS ENABLE A WIDER USE OF HAPLOIDENTICAL ALLOGENEIC TRANSPLANTATION BY REDUCING INFECTION RELATED MORTALITY AND IMPROVING SURVIVAL IN HIGH RISK ACUTE LEUKEMIA.

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Background. Allogeneic transplantation from haploidentical family donors (haplo-SCT) represents the ideal solution to offer to every and each patient with high risk leukemia the potential cure of allogeneic adoptive immunotherapy. However, the delayed immune reconstitution secondary to the required profound T-cell depletion compromise haplo-SCT with a high rate of late mortality and relapse. *Methods.* In a phase II multicenter trial (MM TK007), we explored early add-backs of donor lymphocytes genetically engineered to express the herpes simplex thymidine kinase (TK-DLI) suicide gene after haplo-SCT, in inducing early immune reconstitution and selective control of GvHD by ganciclovir. *Results.* Thirty-one advanced age pts (median age 51, 17-64) were transplanted for high risk leukemia. No immune reconstitution and no graft versus host disease (GvHD) were observed in absence of TK-DLI. 17 pts received TK-DLI at a median dose of 107/kg with 1st infusion at d +42; 14 pts obtained a prompt and sustained immune reconstitution with CD3+ >100/mcl at a median time of 86 d (57-127) from SCT and 21 d (13-42) from TK-DLI. Six pts developed acute (GvHD), (grade I to IV) that was always completely abrogated by ganciclovir. In patients in remission of leukemia at time of SCT, who were alive at day +42 and received TK-DLI, overall survival was 65% at 2 years (intention-to-treat analysis: 46%).The cumulative incidence of TRM and relapse showed a 40% probability of mortality with a median time of death of 90 days and last event at day +166. The cumulative infectious mortality beyond day 100 post transplant was 12.5% in our population, versus 53% of historical data. These data correlate with rapid development of normalization of the T cell repertoire documented by spectratype, followed by detection of high frequencies of T cells specific for CMV and EBV by gIFN ELISpot. *Conclusions.* These results indicate that TK-DLI abolish late mortality after CD34+ haplo-SCT in adults. Overall survival rates in patients treated with TK-DLI were superior as compared to survivals from large populations of haploidentical SCT of EBMT registry database. A phase III randomized multicentric study will start in 2006 to validate prospectively the advantage of TK-DLI in haplo-SCT.

0531

DIFFERENTIAL IMMUNOMODULATION OF ENGRAFTMENT FOLLOWING MHC-MISMATCHED HEMATOPOIETIC STEM CELL TRANSPLANTATION BY COTRANSPLANTATION OF HOST VERSUS DONOR MESENCHYMAL STEM CELLS

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Background. Mesenchymal stem cells (MSCs) are multipotent progenitor cells that have emerged as a promising tool for clinical application. Further clinical interest has been raised by the observation that MSCs are immunoprivileged, and display immunosuppressive capacities. These properties might be of therapeutic value in allogeneic transplantation to prevent graft rejection and for the prevention and treatment of graft-versus-host disease. *Aim.* In the present study, we examined the *in vivo* immunomodulatory properties of MSC in murine models of allogeneic bone marrow (BM) transplantation. *Methods.* Balb/c or C57Bl/6 recipients were sublethally irradiated and transplanted with T-cell depleted bone marrow cells of C57Bl/6 (major MHC mismatched) or Balb/b (multiple minor histocompatibility antigen mismatched) donors, respectively, with or without host, donor or third party MSCs. MSCs were expanded from host, donor or third party BM cells in medium containing FCS. MSCs were used in cotransplantation experiment at passage 7. Chimerism was assessed at various time points after transplantation by flow cytometry. *Results.* The cotransplantation of host-derived MSCs resulted in a significantly increased engraftment rate both in multiple minor mismatched recipients (82% versus 44% in the

absence of MSCs) as in major MHC mismatched recipients (50% engraftment versus 17% in the absence of MSCs). Furthermore, the MSC-facilitated engraftment was still evident at 4 months after transplantation and the donor chimerism included both lymphoid (CD3+, B220+) and myeloid (GR-1+) lineages. In contrast, infusion of donor-derived MSCs was associated with a significantly increased rejection rate of allogeneic donor BM cells in both multiple minor antigen mismatched transplants (44% versus 0% in the presence of donor MSCs) and major MHC mismatched transplants (80% versus 22% in the presence of donor MSCs). Finally, the addition of third party MSCs derived from C3H mice did not affect the engraftment rate of MHC mismatched transplants. *in vivo* cytotoxicity data, employing differentially CFSE labeled splenocytes, showed that the infusion of merely allogeneic donor or third party MSCs in naïve mice was sufficient to induce a memory T cell response. *Summary/Conclusions.* Taken together, these results show that MSCs are capable of modulating immune responses *in vivo* and that these responses are affected by MHC antigen matching between donors and recipients. In addition, MSCS are not intrinsically immunoprivileged and are capable of inducing a memory T cell response following injection *in vivo* in immunocompetent hosts. Following cotransplantation in immunocompromised hosts that have received sublethal irradiation, allogeneic MSCs still induce an allogeneic response resulting in graft rejection. Although it is still unclear whether the immunogenicity of allogeneic MSCs is preserved after a full myeloablative conditioning regimen, the possibility that allogeneic or third party MSCs are immunogenic may be taken into account in designing clinical studies in the setting of allogeneic stem cell transplantation.

0532

SUSTAINED RECONSTITUTION OF NADPH-OXIDASE ACTIVITY IN X-LINKED CHRONIC GRANULOMATOUS DISEASE FOLLOWING RETROVIRAL GENE THERAPY AND NONMYELOABLATIVE CONDITIONING

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Gene transfer into hematopoietic stem cells has been successfully used to correct immunodeficiencies affecting the lymphoid compartment. However, similar results have not been reported for diseases affecting myeloid cells, mainly due to low engraftment levels of gene-modified cells observed in unconditioned patients. Here we report on two adult patients who received gene-transduced hematopoietic stem cells in combination with nonmyeloablative bone marrow conditioning for the treatment of X-linked Chronic Granulomatous Disease (X-CGD), a primary immunodeficiency caused by a defect in the oxidative antimicrobial activity of phagocytes. Therapeutically significant gene marking was detected in neutrophils of both patients leading to large numbers (up to 60%) of functionally corrected phagocytes 16 months after gene therapy. This high correction resulted from an unexpected but temporarily restricted expansion of gene transduced myeloid cells *in vivo*. Gene marking levels in B-cells has remained constant at a level of 20%, while gene marking in T-cells is below 5%. Gene marking in bone marrow was detected at levels between 30% and 40% one year after transplantation. Killing assays *in vitro* have demonstrated antibacterial and antifungal activity in gene transduced phagocytes and both patients recovered of Staph. aureus and A. fumigatus infections after gene therapy. Both patients have been free of severe bacterial and fungal infections since transplantation. Large-scale mapping of retroviral integration site distribution revealed that activating insertions in the zinc finger transcription factor homologs MDS1/EVI1, PRDM16, or in SETBP1 have expanded gene-corrected long term myelopoiesis 3- to 4-fold in both patients, providing direct evidence in humans that these genes influence regulation of normal long-term hematopoiesis. Although it is likely that insertional effects have reinforced the therapeutic success observed in this trial, our results suggest that gene therapy in combination with bone marrow conditioning is a therapeutic option for inherited diseases affecting the myeloid compartment and can be successfully used to treat CGD.

0533

IDENTIFICATION OF THE VON WILLEBRAND FACTOR BINDING SITE IN COLLAGEN USING TRIPLE HELICAL PEPTIDES

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Background. The interaction of the plasma protein von Willebrand factor (VWF) with subendothelial collagen initiates adhesion of blood platelets to the damaged vessel wall or ruptured atherosclerotic plaque. A detailed molecular description of the VWF-collagen interaction may facilitate development of a novel class of antithrombotic drugs that inhibits this vital step in platelet thrombus formation. *Aims.* We have previously used site-directed mutagenesis to map the collagen-binding site in the VWF A3 domain. Here, we aimed to identify the aminoacid sequence in collagen type III which mediates VWF binding. *Methods.* We have synthesized a set of 57 peptides, each containing 27 amino acids of native collagen sequence flanked at each end by five GPP (standard amino acid nomenclature) triplets which support the triple helical structure that is essential for ligand recognition by collagen. The sequence of each peptide overlaps by 9 amino acids with that of each adjacent peptide. VWF binding to these peptides was assessed by a solid phase binding assay. Also, platelet deposition from whole blood under flow to peptides that interact with VWF was assessed. Finally, we used the information obtained from these binding experiments to construct a molecular model of the collagen-VWF interaction. *Results.* A single peptide from this set (#23) was shown to bind VWF in a solid-phase binding assay. The affinity of peptide #23 for VWF was comparable to that of native collagen type III. The peptide #23-VWF interaction was abolished by a monoclonal antibody directed against the collagen-binding site on the VWF A3 domain. Furthermore, recombinant VWF variants that were previously shown to lack collagen-binding capacity (delta A3, His1023Ala) were not able to bind to the peptide. Immobilized peptide #23 also supported platelet adhesion from whole blood under flow conditions. Platelet adhesion to peptide #23 could be abrogated by a monoclonal antibody directed against the VWF A3 domain, which inhibits the interaction of VWF with full-length collagen. We subsequently synthesized a set of truncated and alanine-modified triple helical peptides based on the sequence of #23, which were all tested for VWF and platelet binding from whole blood under flow conditions. Modified peptides either strongly interacted with both VWF and platelets, or lacked both VWF and platelet binding. Based on these experiments, we identified the sequence RGQOGVMGF (O is hydroxyproline) as the minimal VWF binding sequence in collagen type III. Mutation of either Q or M to alanine (A) did not affect VWF binding, whereas replacement of R, O, V, and F by A completely abolished VWF binding. Glycine residues were not replaced, as they are essential for triple helix formation. A model of the VWF A3 domain with this nonapeptide collagen sequence was constructed to give detailed insight into the VWF-collagen interaction. *Conclusion.* We have identified a 9 amino acid sequence in collagen type III that is entirely responsible for high affinity binding to VWF. The detailed molecular description of the VWF-collagen interaction described here may facilitate development of agents disrupting this interaction, which may have potential as antithrombotic drugs.

0534

Pten CONTROLS HEMATOPOIETIC STEM CELLS AND PREVENTS LEUKEMOGENESIS

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Pten (phosphatase and tensin homologue deleted on chromosome ten) encodes a tumor suppressor gene involved in hereditary (Cowden disease) and sporadic human cancers, such as gliomas, endometrial and breast cancers. The Pten phosphatase inhibits the PI3-kinase pathway and plays a key role in apoptosis, cell growth, proliferation and migration. Deficiency of Pten in mice causes early embryonic lethality preventing the analysis of Pten function during development or in the mature organism. Recently it was shown that conditional deletion of the pten gene causes an increase in neuronal stem/progenitor cells due to decreased apoptosis and increased self-renewal (Groszer *et al.*, 2001). Our aim is to study the role of Pten in other self-renewing tissues such as the hematopoietic stem cell compartment. We generated a mouse line in which the pten gene can be deleted in hematopoietic cells including hematopoietic stem cells (HSCs). This was achieved by crossing the conditional pten (ptenflox) allele with the IFN-α inducible MxCre transgene. Cre activity was induced in 4 week old MxCre;ptenflox/flox mice, converting the ptenflox alleles into pten (delta) null alleles in HSCs and other hematopoietic cell types present in the bone marrow. Pten mutant mice show severely enlarged spleens, due to a dramatic expansion of granulocytes, erythrocytes and megakaryocytes. In addition, mutant mice accumulate immature cancer-like cell types and develop transplantable leukemias within 4-8 weeks. Furthermore, normal numbers of HSCs are present in the bone marrow, however the total number of phenotypic HSCs is 6-fold increased in the spleen. Label retaining assays indicate that the quiescent HSC pool in the bone marrow is lost in Pten mutants suggesting that the PI3-kinase pathway controls the transition of HSCs from a long-term quiescent to a self-renewing mode. In summary these results suggest that Pten activity restricts HSC self-renewal, and that it functions as a tumor suppressor in the hematopoietic system.

0535

SAFETY AND EFFICACY OF THE TERMINAL COMPLEMENT INHIBITOR ECULIZUMAB IN A PHASE III TRIAL IN PATIENTS WITH PAROXYSMAL NOCTURNAL HEMOGLOBINURIA

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Background. Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired genetic disorder resulting in the clonal expansion of somatically mutated hematopoietic stem cells with a deficiency of glycosylphosphatidylinositol (GPI)-anchored proteins from the surface of all blood cells. Absence of the GPI-anchored terminal complement inhibitor CD59 from the surface of erythrocytes and platelets results in hemolysis and platelet activation, respectively. Excessive or persistent intravascular hemolysis can result in anemia, fatigue, thrombosis, pain, pulmonary hypertension, poor quality of life (QoL), and frequently a dependency on transfusions to maintain hemoglobin levels. Currently there are no approved or effective therapies that reduce intravascular hemolysis and improve the associated clinical morbidities in PNH. *Aims.* The pivotal phase III clinical study, TRIUMPH (Transfusion Reduction Efficacy and Safety Clinical Investigation, Randomized, Multi-Center, Double-Blind, Placebo-Controlled, Using Eculizumab in Paroxysmal Nocturnal Hemoglobinuria), evaluated the efficacy and safety of eculizumab, a humanized monoclonal antibody against C5 that inhibits terminal complement activation compared to placebo in a cohort of PNH patients. *Methods.* Patients were randomized to receive either placebo or eculizumab administered intravenously at 600 mg weekly for 4 weeks and then 900 mg every 2 weeks commencing the fifth week for a total of 6 months of therapy. The co-primary endpoints were stabilization of hemoglobin levels and reduction in transfused blood units. Prespecified measures of hemolysis and QoL were assessed. *Results.* Eighty-seven PNH patients (39 sites, 11 countries) were randomized 1:1 to receive either eculizumab or placebo. Eculizumab therapy was safe and well tolerated in the study. Both primary endpoints were met with statistical significance: 1) stabilization of hemoglobin levels was achieved by 48.8% of eculizumab-treated patients and by 0% of placebo-treated patients (p=0.000000014); 2) median transfused packed red blood cells (PRBCs) were 0 units in the eculizumab-treated group compared with 10 units in placebo (p=0.0000000006). Fifty-one percent of eculizumab-treated patients were completely transfusion-independent through week 26 (study end), while every placebo-treated patient received at least one transfusion by week 14 (p=0.000000005). Eculizumab treatment dramatically reduced intravascular hemolysis, as evidenced by an 85.8% decrease in the lactate dehydrogenase area under the curve relative to placebo (p<0.00000000001). Concomitantly, eculizumab treatment resulted in an increase in the proportion of PNH type III RBCs from 28.1% at baseline to 56.9% by week 26 while the proportion in the placebo group remained constant (p=0.00005). Fatigue, as measured by the FACIT-Fatigue QoL instrument was significantly improved (p=0.000006), and significant improvements were also demonstrated in QoL subscales of the EORTC QLQ-30 instrument including fatigue (p=0.0000006), global health status (p=0.00002), physical functioning (p=0.000003), emotional functioning (p=0.008), cognitive functioning (p=0.002), role functioning (p=0.0001), social functioning (p=0.003), pain (p=0.002), dyspnea (p=0.0008), appetite loss (p=0.00004) and insomnia (p=0.014). The 2 most common adverse events in the trial were headache and nasopharyngitis. *Summary/Conclusions.* Eculizumab stabilized hemoglobin levels, decreased the need for transfusions and provided clinically meaningful improvements in fatigue and other QoL parameters in patients with PNH through reduction of intravascular hemolysis. Long-term eculizumab treatment in PNH is effective and well tolerated.

POSTER SESSION II

Transfusion medicine

0536

BLOOD-SHOT EYES: AUTOLOGOUS SERUM EYE DROPS FOR GRAFT-VERSUS-HOST DISEASE AND OTHER DISORDERS

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Background. Autologous serum eye drops have been used for treatment of ocular surface disease including chronic corneal ulceration and severe dry eye caused by graft-versus-host disease (GVHD), Stevens Johnson syndrome and Sjogren's syndrome. Autologous serum eye drops are hypothesized to be superior tear replacement to commercially available lubricants because (1) serum contains similar elements to natural tears, such as epidermal growth factor & vitamin A, facilitating corneal healing, (2) immunoglobulins may provide antimicrobial benefit and (3) the autologous source limits antibody formation & allergic reactions, allowing longer term therapy. *Aims.* To provide safe, high quality autologous serum eye drops for treatment of patients with refractory corneal ulceration and dry eye syndromes. *Methods.* Collection, processing and testing of units occurs at the Australian Red Cross Blood Service (ARCBS) GMP-licensed facility. From 2000-2005, eye drops were prepared using aseptic technique from autologous whole blood collected into blood bags or serum separator tubes. Following centrifugation, serum was extracted & separated into 30 mL aliquots for storage in sterile vials at -30°C. When required, aliquots were thawed, diluted with 120 mL sterile 0.9% saline and transferred into sterilised 5mL eye drop bottles. All manipulations were performed in a class II biohazard cabinet using sterile consumables. Since September 2005 we use the following fully closed system: Whole blood donations (up to 450 mL ± 10%) are collected from patients meeting ARCBS autologous donor eligibility criteria. Collection bags without anticoagulant (Baxter trio dry packs) facilitate clotting and serum removal following centrifugation. Serum is diluted to 20% by adding 200 mL sterile 0.9% saline to each 50mL serum via sterile connection. A sample is taken for microbial testing, and the remaining volume transferred into 20 metres tubing of a Macopharma blood bag. Tubing is segmented at ~ 7cm intervals, creating approximately 800 segments (individual doses) from a single donation. These are frozen at < -18°C. Labelled storage containers are stored in local hospital blood bank freezers and/or by patients at home. Patients use the drops according to clinical need in consultation with their doctor. One donation can provide supplies for approximately 6 months. Information sheets for patients, doctors and hospital laboratory staff reinforce appropriate product storage and handling. *Results.* We have collected blood from 52 patients. Clinical indications include GVHD, Sjogren's syndrome/scleroderma, Stevens Johnson syndrome, and epithelial defects following corneal grafts. One patient was excluded (hepatitis B positive on routine viral testing) and one set of drops, prepared using the earlier method, was discarded for a positive microbial culture. No complications of therapy have been reported. Some patients require therapy for short-term indications, others have used the drops for extended periods, the longest being nearly 6 years. The 20% serum dilution, chosen in 2000 based on available literature, appears optimal to prevent eye crusting while maintaining adequate potency and a practical interval between autologous blood donations. *Summary/Conclusions*. Our method for collection and preparation of autologous serum eye drops appears safe and clinically effective. This therapy can help alleviate symptoms of acute and chronic eye surface disease, including GVHD.

0537

ERYTHROID CRISIS CAUSED BY PARVOVIRUS B19 TRANSMITTED VIA RED BLOOD CELL TRANSFUSION - ITS DIRECT DETECTION BY PCR DIRECT SEQUENCING METHOD

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Background. The administration of blood products possesses certain risk of transmission of a variety of viruses including parvovirus B19. Parvovirus B19 (B19) is a small no enveloped erythrovirus that can cause various clinical manifestations. B19 infection has been reported to cause erythroid crisis in immunocompromised hosts including patients with AIDS, malignancies, or patients undergoing chemotherapy or organ transplantation. The virus is normally transmitted via respiratory tract; however, transmission via the administration of blood products has also been speculated. The infectivity of B19 in the blood products is affected by the level of anti-B19 IgG in the products, as well as recipient immune status. Therefore, it is often difficult to prove directly whether the B19 transmitted via blood transfusion does cause erythroid crisis. We here report a case of erythroid crisis after red blood cell (RBC) transfusion, in which we could successfully detect the same B19 virus as in the blood product. *Patient.* A 41-year-old Japanese man was admitted to our hospital for the treatment of hairy cell leukemia in May 2005. He was treated with cladribine (0.09 mg/kg/day) for 7 days. 16 days after the treatment, hairy cells in the peripheral blood became undetectable, but the patient became anemic and received 2 units of RBC transfusion. He remained anemic with reticulocytopenia even after the recovery of granulocytes. The diagnosis of B19 associated with PRCA was made according to the presence of B19-specific IgM antibody and viral DNA in sera. To assess whether the B19 was transmitted via the blood product, we performed PCR direct sequencing analysis of B19 in the patient and his blood donor's sera. The DNA sequence of 2 distinct regions of the genome in B19 virus Ns1 and Vp1 were amplified and then directly sequenced. Sequencing of Ns1 and Vp1 regions of B19 virus from the patient was completely corresponded to those of blood donor serum. The results suggest that B19 virus is horizontally transmitted from the blood products, and it may be the cause of erythroid crisis in the patient. The patient was treated with intravenous immunoglobulin (5 g/body for 2 days) without any response. Erythropoiesis of the patient began to recover spontaneously around 50 days after the treatment, and B19 virus DNA became negative by PCR analysis. *Conclusion.* This is the first case report demonstrating the transmission of B19 via RBC transfusion did cause erythroid crisis in the recipient, by using genomic PCR direct sequencing method. Blood products containing B19 DNA may possess a potential risk, especially for immunocompromised patients. Therefore, more sensitive screening for detecting B19 virus should be applied especially for transfusion in these patients.

0538

USE OF TAGVHD WARNINGS IN PATIENTS RECEIVING PURINE ANALOGUE CHEMOTHERAPY

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Background. Transfusion associated graft versus host disease (TaGVHD) is a rare but serious complication of blood transfusion with mortality rates >90%. It occurs following the transfusion of immunocompetent donor lymphocytes. These engraft and proliferate in a susceptible host causing widespread tissue damage. TaGVHD can be prevented by γ irradiation of cellular blood components for patients in at risk groups. A major risk group is patients who have received purine analogue chemotherapy, principally Fludarabine. The annual SHOT report demonstrates that *incorrect component transfused* remains the commonest transfusion error and many of these are accounted for by not selecting irradiated blood. *Aims.* To find if patients who had received Fludarabine had 1) a BCSH/NBS TaGVHD sticker in their notes 2) a flag on the hospital transfusion laboratory (HTL) IT system indicating the requirement for irradiated blood 3) a warning in the nursing profile regarding the requirement for irradiated blood. An additional aim was 4) to confirm consistency of results at a second site. *Methods.* 1) Patients who had received Fludarabine chemotherapy were identified via pharmacy records. 2) Patient case records were traced and inspected for a BCSH/NBS TaGVHD sticker or other warning indicating the need for irradiated blood. 3) The HTL IT system was examined for a flag indicating the requirement for irradiated blood and checked to determine if blood subsequently transfused had been irradiated. 4) Nursing profiles for the patients were traced and examined for comments on the requirement for irradiated blood. *Results.* 1) Case records were obtained for 28/34 patients at site 1 and 14/14 patients at site 2. 2) At site 1 only 7/28 (25%) had any warning for the requirement for irradiated blood and only 2 (7%) had the BCSH/NBS TaGVHD sticker. At site 2 all 14 (100%) case records contained a warning regarding the requirement for irradiated blood, however, 0/14 had the BCSH/NBS sticker. 3) Only 1/34 patients (site 1) and 1/14 patients (site 2) did not have a flag on the HTL IT system regarding the requirement for irradiated blood and neither of

these patients had been transfused. All cellular products transfused subsequent to Fludarabine therapy had been irradiated. 4) 21/34 nursing profiles were inspected (site 1) and 14/21 had some indication of the requirement for irradiated blood. Site 2 has a unified nursing and medical case record. *Conclusions.* 1) The high rate of warning flags in The HTL IT system indicates that medical and/or biomedical staff are aware of the need for irradiated blood in these patients. 2) The BCSH/NBS stickers are not being used and it would seem likely that patients are unaware of this potential risk from transfusion. 4) Although transfusion within the patient's institution may be safe, results indicate that transfusion at another site is likely to be potentially hazardous. We suspect that these findings are not unique to these 2 centres. The findings of this study have led us to alter our practice with pharmacy staff distributing the BCSH/NBS patient information leaflet and sticker at the time of first Fludarabine prescription.

0539

FACTORS DETERMINING THE RISK OF SEVERE (WHO GRADE 3 AND 4) HEMORRHAGE IN HEMATOLOGIC PATIENTS

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Current indications for platelet transfusion in the management of thrombocytopenia hinge on studies, that used minor bleeding as the end point. Minor bleeding may not be a good correlate for life-threatening hemorrhage, that poses real risk of death from that cause. Therefore, we have attempted to verify these indications through evaluation of severe hemorrhages. In this study, we have analyzed retrospectively circumstances of 146 severe hemorrhages (20 grade 3 hemorrhages, and 126 grade 4, among them 109 fatal) that have occurred among 1590 patients hospitalized because of various hematologic disorders with a goal to identify factors that might have contributed to the occurrence of hemorrhage. It was found that unintentional violation of platelet transfusion policy (no transfusion for patients with platelet count below 10×10^9/L) might have been responsible for 8 such hemorrhages, at the most. Similarly, 8 hemorrhages have occurred in patients with normal or increased platelet count. Frequency of remaining 130 hemorrhages was inversely correlated with platelet count. Tendency for increased number of hemorrhages started with platelet count below 50×10^9/L, became significant below 40×10^9/L, and further increased until below 20×10^9/L with plateau afterwards.The highest frequency of hemorrhages was in patients with various forms of acute leukemia, either primary or secondary and aplastic anemia (18-60% of all patients with given form of disease had severe hemorrhage) and was much lower in various lymphomas (between 1 and 6%). Moreover, almost half of hemorrhages in acute leukemia has occurred in patients with early disease (within 50 days of diagnosis). The lowest frequency of hemorrhages was for ITP, when only one hemorrhage among 72 patients has occurred. For patients with platelet count between 20 and 50×10^9/L concomitant presence of plasma clotting factor abnormalities was an important factor contributing to the occurrence of hemorrhage. Unexpectedly, the presence of severe infection had no effect on hemorrhage occurrence in that group. These data may suggest that in order to effectively prevent life-threatening hemorrhages in patients with early acute leukemia it may be necessary to increase transfusion threshold to at least 20×10^9/L, in this group of patients only. Moreover, the lower number of grade 3 than grade 4 hemorrhages in that cohort may suggest that there is a disproportion between benign and severe hemorrhages in thrombocytopenic patients possibly related to the undefined differences in the resistance of blood vessels in particular patients to the injury.

0540

LONG-TERM ERYTHRO-EXCHANGE IN THE TREATMENT AND PREVENTION OF SEVERE SICKLE CELL DISEASE RELATED COMPLICATIONS IN SICILY

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Background. Sickle Cell Disease (SCD) is a severe health problem in Sicily. Among the Sicilian population some individuals have frequent vaso-occlusive complications, whereas others have sporadic/mild episodes of SCD crises. Although the complications associated with SCD are numerous, both direct and indirect, essentially all are associated with recurrent vascular occ1usions. If the goal of therapy is to increase the capacity for oxygen transport and drastically reduce the levels of HbS, the therapy should be erythro-exchange and not simple transfusional therapy. Erythro-exchange is indicated for the treatment of various severe complications of SCD, such as acute pulmonary syndrome, stroke, and acute syndromes involving multiple organs. In this study, from November 1999 through December 2005, we investigated a selected group of patients affected by SCD who developed one or more severe SCD-related episodes. The aim was to evaluate if a long-term exchange treatment would effectively prevent or reduce the recurrence rates of SCD complications, and to compare this management program with exchange therapy disadvantages. *Materials and Methods.* We studied 9 patients affected by SCD, 6 ma1es and 3 females with a mean age of 38.2 years (range 24-62 years); informed consent was obtained from all subjects. A1l patients refused Hydroxyurea treatment for personal reasons. The genotypic classification included 4 patients with HbSβ+, and 5 with HbSS. Two Out Of The 9 Patients Developed One Stroke Episode, Five Acute Chest Syndrome, And Two Recurrent And Severe Vaso Occlusive Episodes. Acute Episode Treatment: analgesic regimen, hydration, oxygen therapy, antibiotics associated with early erythro-exchange. A second exchange was performed after 3-4 days to stabilize HbS levels between 20% and 35%. Long-Term Care And Follow-Up: exchange transfusion was applied therapeutically as a prophylactic regimen and the goal of the protocol was to maintain an HBS of ≤55% and ≈ 35% pre and post pheresis. Transfusion guidelines include the use of units matched with an extended phenotype. Patients were monitored for alloantibodies and transmission of viral diseases 30 days after each exchange for the first six months and twice per year during follow-up (4-5 years). Red cell exchange was performed using a COBE (Lake-wood CO) Spectra TM continuous flow system. *Results.* the mean pre and post haemoglobin was 9.5±0.85 g/dL and 11.1±0.93 respectively; haematocrit 29.5±2.91% and 33.7±4.31%; ferritin levels were 2338.4 ng/mL pre and 1954 ng/mL post exchange. All patients had a dramatic clinical improvement within 2-4 days of the procedure. During follow-up, none of the 9 patients developed iron overload, viral complication related to transfusion therapy, allo-immunization or recurrence of SCD complications. *Discussion.* in conclusion we can say that this procedure provided a dramatic resolution of SCD episodes and long-term therapy in our patients. although there is the risk of recurrence, this program has proved to be very useful in treating SCD related complications. Finally, in the light of our results, we believe that this therapeutic approach could improve both the length and quality of life for SCD patients.

0541

HAEMOVIGILANCE FOR THE TRANSFUSION THERAPY OF PATIENTS WITH THALASSAEMIA

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Background. Multi-transfused patients are exposed to an increased risk of developing alloimmunization and other immune or non-immune mediated transfusion-associated adverse reactions as well as contracting transfusion-transmitted infections. *Aims.* Against this background, haemovigilance systems offer useful information about current practices both in the laboratory and in the clinical setting of transfusion, as well as a tool for evaluating quality management procedures and providing blood safety indices. In this way, quality improvement may be monitored at national and local level, to the benefit of all patients in need of transfusion as well as those whose life depends on regular transfusion therapy. *Methods.* In the context of the Hellenic haemovigilance system initiated in November 1995, we analyzed immune, non-immune and infectious adverse reactions and adverse events associated with the transfusion of red cell concentrates (RCCs) in patients with thalassaemia. We examined numerator data, such as the absolute number of adverse events/adverse reactions reported in 1997-2004 during or after transfusion. These data were analyzed by the type of reaction, severity, imputability and morbidity. Apart from iron overload - identified as the predominant complication of multi-transfusion with RBCs - we studied the incidence of non-haemolytic febrile reaction (NFHTR) in relation to leukoreduction (pre-storage, bedside and laboratory post-storage), the transfusion reaction rate (TRR) and the patients' reaction rate (PRR). Acute and delayed haemolytic reactions and the incidence of alloimmunization and autoimmunization were also investigated and analyzed in relation to red cell antigen-matching policy. Transfusion of incorrect RBCs, TRALI, TA-GvHD, allergic and anaphylactic reactions, infectious

and other adverse reactions were also examined. The data were than analyzed in relation to the number of transfusions (units of RBCs). *Results.* NHFTR incidence was 0.37% of blood units. TRR was 0.37% and PRR 7.2%. Further analysis showed that the TRR was 0.2% and PRR 1.8% in patients transfused with pre-storage leukodepleted RCCs, compared to 0.8% and 5.2% respectively with bedside filtered RCCs. Delayed haemolytic reaction was diagnosed in 47 patients (64% had one antibody, 23% two antibodies and 13% multiple antibodies). Alloimmunization was diagnosed in 3.5% of the patients. The most common antibodies detected were of the Rh system, JKa, Kpa, Leα+β, Fyα+β and S. Autoimmunization of the IgG type was diagnosed in 1.5%. One patient with S-thal died of hyperhaemolysis syndrome (DAT positive of IgG type, anti-C3 and anti-C3d present) in the year 2004. The seroprevalence of HBsAg was 1%, of anti-HCV 60%, of anti-HIV 1% and of anti-HTLV 1%. The residual risk for HIV in this group of patients is 1:1.500.000 units. Yersinia enterocolitica and Klembsiella have been reported in 13 and 2 patients, respectively, and Malaria falciparum in 2 patients. *Conclusion.* Alloimmunization and autoimmunization were low in relation to the number of blood transfusions, while hyperhaemolysis caused the death of one S-thal patient. TRR and PRR due to NHFTR were significantly lower in pre-storage leukodepleted blood compared to bedside filtration. These haemovigilance data show that further improvements are necessary in leukoreduction, RBC washing and phenocompatibility policies.

Table 1. Haemovigilance data for thalassaemic patients during 1997-2004.

Number of patients	Issued RBCs	Acute haemolysis	Incorrect component transfused	Delayed haemolysis	NHFTR	Allergic/ Anaphylactic	TRALI	Infections	Others
1336*	292,599*	9	0	67	588	521	0	17	134
		0.7%	0	5%	44%	39%	0	1.3%	10%

*46% of total; **18,5% of total.

0542

PROGRESSIVE MEMBRANE AND CYTOSKELETON ACCUMULATION OF OXIDIZED/DENATURED HEMOGLOBIN IN STORED RED BLOOD CELLS

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Background. In conditions of increased cellular stress, the submembrane cytoskeleton of the red blood cell (RBC) sustains certain modifications. During storage in anticoagulant media, red cell membrane proteins undergo progressive alterations such as pathological interactions, oxidation, crosslinking and increased hemoglobin association. Previous studies have revealed increased RBC membrane and cytoskeleton protein carbonyls with prolonged storage in CPDA blood bags. The Hb composition of the cytoskeletons extracted from RBC membranes in the course of the storage period in CPDA remains unclear. *Aims.* To determine whether the storage-induced membrane bound of Hb is in denatured/oxidized form and whether this Hb-accumulation defect concerns the skeletal proteins as well. *Methods.* RBC concentrates in CPDA from six eligible blood donors were prepared according to the standard operating procedures. Each RBC unit was followed up during the storage period of 35 days and shortly afterwards. Membrane skeletons were prepared by Triton X-100 extraction of ghosts. The membrane ghosts and skeletons of days 0-2 of these units, in addition to fresh preparations from ten healthy subjects, were used as controls. Total membrane ghosts and cytoskeletons were analyzed by SDS-PAGE densitometry and immunoblotted against monoclonal and polyclonal human Hb-specific antibodies. *Results.* As expected, the Hb was increasingly associated with the membrane in proportion to the duration of storage. Apart from the bands of globin monomers, a considerable proportion of the membrane-bound globin species consists of nonreducible crosslinkings of probably oxidized/denatured hemoglobin or hemichromes. The same storage effect of Hb concerns the cytoskeletons of the stored RBCs as well. The immunoblotting analysis of the Triton insoluble cytoskeletons, revealed a progressive accumulation of Hb and crosslinked multimers to them in amounts proportionate to the age of storage. *Summary/Conclusions.* The storage of RBCs in CPDA triggers the accumulation of Hb and hemichromes in the ghost membranes and cytoskeletons. The formation of similar globin complexes in severe thalassemia, sickle cell disease and in senescent RBCs is dictated by the increased oxidative stress and is positively correlated with increased cell density and membrane rigidity that are also evident in RBC storage. These data corroborate the evidence for the occurrence of oxidative damage in membrane proteins of stored RBCs and suggest the possible use of antioxidants in the RBC stored units intended for transfusion. They partially address the pathophysiological mechanisms underlying the RBC storage lesion and add some new insight in the field of RBC storage as a cytoskeleton-associated pathology.

0543

MEMBRANE AND CYTOSKELETON PROTEIN CARBONYLATION IN NON-LEUKODEPLETED CPDA-PRESERVED RED BLOOD CELLS

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Background. Despite the arrest of the normal aging process, *ex vivo* storage causes a number of reversible and irreversible biochemical and mechanical changes to the red blood cells (RBCs) and accumulation of bioactive substances in storage medium, collectively referred to as *storage lesion*. Some of the negative effects of RBC transfusion are associated with the storage lesion. The importance of RBC oxidative damange in the storage lesion is not well documented. *Aims.* To determine the possible storage-induced membrane and cytoskeleton protein oxidation in CPDA-preserved non-leukodepleted RBCs bags in the course of transfusion period storage. *Methods.* RBC concentrates from six eligible blood donors were prepared according to the standard operating procedures. Each RBC unit was followed up during the storage period of 35 days and shortly afterwards. Membrane skeletons were prepared by Triton X-100 extraction of ghosts. The membrane ghosts and skeletons of days 0-2 of these units, in addition to fresh preparations from ten healthy subjects, were used as controls. Total ghosts and membrane skeletons were analyzed by SDS-PAGE densitometry and immunoblotted against a variety of erythroid-specific antibodies. Carbonylated protein content was determined following 2,4-dinitrophenylhydrazine derivatization and SDS-PAGE coupled with Western blotting. *Results.* Immunoblotting with dinitrophenol-specific antibody revealed increased RBC membrane and cytoskeleton protein carbonyls with prolonged storage in CPDA blood bags. A quantitative and statistical important difference in carbonylation was detected in membrane and cytoskeleton proteins isolated from RBCs stored for different time periods. In comparison to control membranes, there was an evident increase in the number and the intensity of the carbonylated protein bands appearing in the immunostained gel, ranging from MW 240 kDa to 15 kDa. The membrane skeletons stored even for long times in CPDA did not exhibit signs of severe proteolysis, as confirmed by immunoblotting analysis of spectrin, actin and 4.1 proteins. *Summary/Conclusions.* We conclude that the protein carbonylation of RBC membrane and cytoskeleton during banking in CPDA is increased probably in association with the diminution in total antioxidant activity of RBCs. Since the stored RBCs convey less glucose to the pentose phosphate pathway, due to the subsequent decrease in NADPH and ATP levels, there are expected to be less protected against oxidative stress. The specific carbonylation of a set of RBC membrane and cytoskeleton proteins with prolonged storage in CPDA is shown for the first time and supports the concept of protein oxidation as a part of storage lession. These data could give additional, useful information in evaluating improved conditions for storage of RBCs intended for transfusion.

0544

PLATELETS RECOVERY AND TRANSFUSION NEEDS AFTER REDUCED INTENSITY CONDITIONING ALLOGENEIC PERIPHERAL BLOOD STEM CELL TRANSPLANTATION

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Few data are currently available regarding platelets trasfusion needs and the kinetics and predicitive factors for platelets recovery after RIC allo-SCT. In this study, we analyzed the profile of platelets recovery and transfusion needs in the first 100 days after sibling PBSC RIC in a single institution series of 166 consecutive transplantations. Patients and graft characteristics were: age 49 y. (range: 18-70), diagnoses: 66 myeloid malignancies (40%), 64 lymphoid malignancies (39%), and 36 metastatic solid tumors (21%). 112 pts (67%) received an ATG-based RIC, while 54 pts (33%) received a low dose irradiation-based RIC. 75 pts (45%) developed grade 2-4 acute GVHD. Platelets recovery (>20 G/L) was

observed at a median of 9 days (range: 0-99). The kinetics profile of platelets recovery is shown in the figure below. In the whole study population, the nadir was observed around day +7 after allo-SCT, and a plateau was reached about day +35. Filtered and irradiated donor apheresis platelets were used and patients needed a median of 1 unit (range: 0-53). In this series, 83 pts (50%) did not require any platelets transfusion during the follow-up period (median follow-up: 442 days) and 83 patients (50%) received at least one transfusion of platelets (54 were not transfused beyond day +100 after allo-SCT). Platelets count prior to RIC allo-SCT (median count 144 G/L; HR 0.44 (0.28-0.7) p=0.002), conditioning regimen (use of ATG; HR 1.86 (1.08-3.2) p=0.025) and the occurrence of acute (HR 1.54 (1.17-2.01); p=0.001) and severe GVHD (HR 2.36 (1.38-3.05) p=0.0006; 82% of patients with grade 3-4 acute GVHD were transfused) were the parameters significantly associated with platelets transfusion needs in Multivariate analysis. In this cohort, 145 pts could be assessed for platelets recovery at day +100: among them, 99 (68%) had a platelet count >99 G/L. Univariate analysis found a significant impact of AGVHD (p<0.0001) and Platelet count prior conditioning (p=0.012) but only acute GVHD (HR 5.52 (2.48-12.25); p=0.001) was associated with a delayed platelet recovery in a multivariate model. No impacts of pathology, GVHD prophylaxis regimen or CD34+ cell dose were demonstrated. Overall, these observations show a significantly lower rate of platelets transfusions and a quicker kinetic of platelets recovery after RIC allo-SCT and point out the effect of acute GVHD. In addition, considering the low level of myeloablation observed, RIC could be an appropriated field of investigation for the testing of megakaryocytic stimulating agents, towards further improving the safety and outcome of RIC allo-SCT.

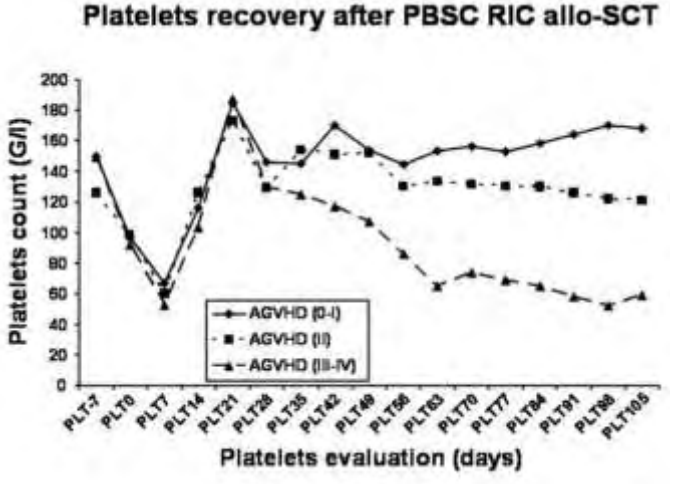

Figure 1. Analysis of Platelets recovery.

0545

CHANGES OF HAEMOSTASIS INDUCED BY LDL-APHERESIS

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Background. Familial hypercholesterolaemia and familial combined hyperlipidaemia are genetic disorders, which are associated with high incidence of severe cardiovascular complications. Extracorporal elimination is used for selective removal of LDL-cholesterol in severe hypercholesterolaemias as combined strength of conservative and invasive lipid-lowering therapy may reduce progression of atherosclerosis in these high-risk patients. Activity of haemostasis plays an important role in the development of atherosclerotic complications. *Aims.* We hypothesize that LDL-apheresis reduces total plasma cholesterol and partially improves haemostasis too. The aim of this work was to verify this hypothesis. *Methods.* Repeated LDL-apheresis procedure (treatment interval 17,5±1,6 days) based on immunoadsorption has been used to treat nine patients with primary hypercholesterolaemia. Primary device Cobe-Spectra (USA); secondary device ADA (Adsorption-desorption automat, Medicap, Germany) with adsorbers Lipopak (Pocard, Russia). To assess the changes of lipid metabolism and haemostasis we analysed many markers - in this branch of our study we measured plasma concentration of thrombomodulin (Asserachrom Thrombomodulin), von Willebrand factor (STA LIAtest vWF), t-PA (Asserachrom t-PA), PAI-1 (Asserachrom PAI-1) and fibrinogen (Fibri-Prest automate 5). We compared plasma concentration of all the above items before and after LDL-apheresis. In long-term monitoring we compared plasma concentrations before LDL-apheresis. All results were evaluated as proportional differences with software Statistica 6.0 (StatSoft Inc., Tulsa, USA). *Results.* LDL-apheresis procedure induced a significant interrelated decrease of total plasma cholesterol, thrombomodulin (-29.1%), von Willebrand factor (-15.1%) and fibrinogen (-21.7%). We have found no significant changes of all the above-mentioned markers in long-term monitoring (the levels of markers were compared before procedures during the period of 300 days). *Summary/Conclusions.* Therapeutic LDL-apheresis is an invasive and effective method, which not only reduces total plasma cholesterol but also partially improves impaired haemostasis too.

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0546

METABOLIC MARKERS AND FUNCTIONAL PARAMETERS OF PLATELET CONCENTRATES COLLECTED BY MULTICOMPONENT APHERESIS WITH TWO DIFFERENT CELL SEPARATORS

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Background. In the recent years the demand for blood components is constantly increasing, while exclusion criteria for donors are strengthened. With multicomponent collection (MCC) we are able to produce several standardized components during one blood donation session. *Aims.* In the present study we investigated platelet (plt) function and metabolic parameters in double plt concentrates (PC) collected by MCC addtitionally to a packed red blood cell (PRBC) concentrate by two devices with different collection modes. *Methods.* 15 donors were randomly allocated to either the TRIMA Access (Gambro BCT) or the AMICUS (Baxter) device and vice versa in the second procedure following a time interval of at least two months. The separators were programmed to collect 6×10^{11} plts (2 units) and one unit of PRBC. Sample collections and analyses were done on day (d) 0 (donation day), d2 and d7. We determined blood cell counts (Sysmex SE-9500, Müller), metabolic markers (Omni, Roche), LDH (Dimension Xpand, Dade Behring) and visual plt quality (swirling effect). Activation of coagulation was performed by INTEG Assay on the ROTEM Coagulation Analyzer (Pentapharm GmbH). To assess specific function of plts in clot formation the assays were repeated by addition of abciximab (Reopro, Centocor B.V.) and cytochalasin D (Sigma Aldrich). Maximum clot firmness (MCF) and difference of maximum clot elasticity (_MCE) were calculated. Assays of hypotonic shock response (HSR) were performed on the SPA 2000 (Chrono-log). *Results.* Plt yields on d0 were 2.79±0.23 and 2.70± 0.54×10^{11}/unit (Trima-PC and Amicus-PC). Metabolic markers are shown in the Table.

	Day 0	Day 2	Day 7
pH			
Amicus-PC	7.05 ± 0.06	7.15 ± 0.13	6.92 ± 0.15
Trima-PC	7.17 ± 0.11*	7.30 ± 0.07*	7.06 ± 0.12*
Glucose (mmol/L)			
Amicus-PC	288.00 ± 22.06	245.75 ± 20.03	144.13 ± 22.62
Trima-PC	309.29 ± 29.78*	284.64 ± 27.41**	183.00 ± 36.85*
Bicarbonate (mmol/L)			
Amicus-PC	19.02 ± 3.17	13.49 ± 2.19	5.89 ± 2.79
Trima-PC	19.07 ± 1.88	14.21 ± 1.33	6.65 ± 1.87
Lactate (mmol/L)			
Amicus-PC	3.99 ± 1.87	8.73 ± 1.64	18.03 ± 3.15
Trima-PC	2.17 ± 0,62*	5.80 ± 0.93**	15.73 ± 4.82*
Potassium (mmol/L)			
Amicus-PC	3.36 ± 0.35	3.41 ± 0.28	3.60 ± 3.79
Trima-PC	3.17 ± 0.23	3.21 ± 0.16*	3.40 ± 0.19
LDH (U/L)			
Amicus-PC	406.63 ± 71.44	492.63 ± 124.43	572.81 ± 147.67
Trima-PC	298.50 ± 51.75**	359.21 ± 46.26**	394.86 ± 51.34**

Values as mean ± SD, * p<0.05, ** p<0.001

Results of thrombelastography showed a statistically significant difference (p<0.05) only on d2: MCF 72.64±2.34 (T-PC) and 69.88±2.6 (A-PC); MCE 243.21±26.41 (T-PC) and 209.88±26.43 (A-PC); during storage the results of thrombelastography did not change significantly. Results of HSR showed a significant difference (p<0.05) between the two

groups only on d0: 59.58±12.31% (T-PC) and 36.66±17.13% (A-PC). HSR increased significantly from d0 to d2 for the group of A-PC (d2: 53.55±15.47) and decreased significantly from d2 to d7 in the T-PC (d2: 62.86±9.05%, d7: 51.96±12.40%). Swirling effect was observed over the entire time period for all products although 2 A-PC showed aggregates on d0. *Conclusion.* The mean plt yield on d0 of the T-PC and the A-PC showed no significant difference (p=0.59). Metabolic markers were well maintained in both groups, although pH, glucose and bicarbonate were partially significantly lower, lactate, potassium and LDH partially significantly higher in the A-PC than in the T-PC. Thrombelastography of the T-PC showed significantly better *in vitro* function parameters only on d2, so did the HSR results of T-PC on d0. If the partially improved *in vitro* metabolic and functional parameters of the T-PC are of *in vivo* relevance has to be evaluated in clinical trials.

0547

IVIG AND RITUXIMAB ALLOWS SUCCESSFUL SOLID ORGAN TRANSPLANTATION IN PATIENTS WITH A POSITIVE CROSSMATCH AND DONOR SPECIFIC ANTI-HLA ANTIBODIES

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Background. Intravenous immunoglobulin (IVIG) in high doses is used increasingly for immunomodulation of various disorders. An illustrative case is end-stage renal failure (ESRF) where until recently, transplantation of dialysis-dependent patients with a positive cytotoxic crossmatch (XM) associated with donor specific anti-HLA antibodies (DSAb) was not possible due to the high incidence of severe acute humoral rejection. Up to 20% of ESRF patients have DSAb, potentially excluding them from transplantation. However, with the use of low dose IVIG with plasmapheresis (PP), or high dose IVIG alone, successful transplantation of such patients has been reported recently (Jordan *et al.,* 2003). Rituximab has also been postulated as appropriate treatment of antibody-mediated rejection (AMR) in this context. *Aims.* To demonstrate that HLA-mismatched kidneys can be successfully transplanted using immunomodulation with IVIG and PP, and to evaluate the role of rituximab in AMR of such cases. *Methods.* Crossmatches were performed by complement dependent cytotoxicity (CDC) on potential donor and recipient pairs. If positive with T &/or B cells with demonstrable DSAb as defined by a combination of CDC and solid phase assay (ELISA, LuminexÒ), recipients commenced mycophenolate mofetil a week before transplantation, PP five days before surgery and subsequently tacrolimus. IVIG 2 g/kg was given 48 hours pre-transplant and one month after transplantation. Any transplant that demonstrated subsequent AMR was treated with PP, IVIG and intravenous rituximab 375 mg/m^2. *Results.* We describe 6 successful renal transplants in which a positive XM with DSAb was overcome by pre-transplant PP, and high dose IVIG. 4 patients had HLA Class I DSAb, 2 had both both HLA Class I and II DSAb. Graft and patient survival is 100% in our patients at a mean of 11 months follow-up (range 8 to 20). Two patients developed AMR treated successfully with PP/IVIG (0.1 g/kg) and one dose of rituximab 375 mg/m^2. The renal function of the transplanted organs is excellent; creatinine and eGFR equivalent or better than donor (~70 mL/min/1.73m). There have been no episodes of cellular rejection, opportunistic infections, or post-transplant diabetes mellitus. *Summary/Conclusions.* XM positive transplantation can be performed successfully and safely with a regimen of PP, high dose IVIG and lower doses of conventional immunosuppression. Rituximab, together with IVIG and PP is successful treatment in episodes of AMR in these patients. This reinforces the important role of IVIG and rituximab in modern renal transplantation programs, and may be extended to other solid organ transplant programs in which the haematologist may be involved.

0548

TRANSFUSION ASSOCIATED GRAFT VERSUS HOST DISEASE IN FOUR IMMUNOCOMPETENT PATIENTS

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We observed transfusion associated graft versus host disease (TA-GVHD) in four male patients (ages: 61, 52, 56 and 57 years). All of the four patients showed the typical clinical and laboratory findings of TA-GVHD, as follows: high fever, diarrhea, erythrodermia, hepatitis and pancytopenia. Oral mucositis was also observed in three patients. Skin biopsies performed in all the patients and were compatible with GVHD microscopic findings. Bone marrow aspiration and biopsy were performed in three of the patients and revealed hypocellularity. There are some other clinical similarities between our patients. All of the patients were immunocompetent, they have received fresh, non-irradiated whole blood from their children or cousins. Transfusion indications of the patients were coronary artery bypass grafting (CABG) surgery in three patients and upper gastrointestinal bleeding in one patient. The symptoms had begun in median 11 days, and the patients died in median 21 days with multiorgan failure. Broad spectrum antibiotics, corticosteroids and packed red cell and platelet transfusions were used given in all patients. Cyclosporine A and chloroquine treatment were also used in two of the patients. HLA typing and sex chromatin analysis using FISH method were done in peripheral blood lymphocytes of the first patient and one of his daughter. The results demonstrated the presence of a donor homozygous for HLA-B65, HLA'DR1, 01 haplotypes and of a chimerism for sex chromatin in this patient's blood. These findings confirmed that the engraftment was supplied by one of the daughters of the patient. In the second patient we extracted and stored DNA by using patient's hair and donor's peripheral lymphocytes. The other two patients could not be studied for HLA antigens because of sudden death events. *Summary*. TA-GVHD is rare, but nearly always fatal complication of blood transfusion. Although immunocompromised patients have an important risk factor for the TA-GVHD, it can also be developed in immunocompetent individuals. Cardiovascular surgery, transfusions from in-family blood donors, genetic homogeneity that can be increased by consanguinity are all the risk factors for occurrence TA-GVHD. There is some common extended major histocompatibility haplotypes in white populations, and the chance of transfusion from a donor homozygous at a particular HLA locus to a patient heterozygous at the same locus, reported as 0,21 percent for one transfusion. This probability will be raised with respect to transfusion number and in the presence of the parental consanguinity. *Conclusions.* TA-GVHD can easily be misdiagnosed as drug reactions or viral infections that may have similar clinical and laboratory findings. Because of the ineffectiveness of treatment opportunities, prevention of TA-GVHD is of paramount importance. Patients at risk should be identified and transfused with irradiated cellular blood products.

0549

THE CELLULAR COMPONENTS INDUCED IMMUNOSUPPRESSION VIA REGULATORY T CELLS IN ALLOGENEIC TRANSFUSION

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Background. Transfusion save a lot of patients by supply of blood components, although there are several tolerable adverse effects. However, some clinical data showed that transfusion might be induced high risk of post-operative infection and higher relapse or mortality rate in cancer patients. There is controversial for relation between transfusion and immune dysfunction. *Aims.* We investigated whether immune dysfunction might be induced after transfusion of cellular components. *Methods.* We used 5 weeks old male BALB/c (H-2d, recipient), female C3H/He (H-2k, donor), and C57/BL (H-2b, third party). We obtained irradiated spleen cells (SP) from BALB/c or C57/BL, and injected to C3H/He mouse via tail vein with intraperitoneal IL-2 administration. Some mice received consequent injection with same condition for 2 days. After 24 hours, we obtained bone marrow (BM), thymus, and SP. For mixed lymphocyte proliferation (MLR), we used irradiated BALB/c SP, as stimulator, and SP from treated C3H(B-C3H), C57 (C-C3H), and not treated donor (control). For analysis of immune cells, we analyzed cell surface markers for each samples. Also, we evaluated cytotoxic effects against A20, YAC-1 cell line after transfusion. *Results.* After 24 hours of transfusion, there were shown profound decrease of cell proliferation and, in some ratio, specific for H-2 complex. Two day transfusion did not show inhibition of cells but proliferation. For inhibitory effects of transfusion, we performed MLR with mixture of control and B-C3H SP. B-C3H SP were induced inhibitory effects according to mixture ratio. There was high level of IL-10 in supernants from mixture with control and B-C3H SP. Also, there were markedly increased CD4+CD25+ cells in BM, SP, and thymus with no change of other immune cells after 24 hours. However, in 2-day treated cases, there were increased some adhesion molecules and co-stimulatory markers. In cytotoxicity, SP after transfusion did not have cyto-

toxic effects against A20, YAC-1 cells. *Conclusion.* We suggested that cellular components in transfusion might be contributed some immune regulatory effects by CD4+CD25+cells just after 24 hours. Therefore, we have to consider patients' immune state after transfusion, in view of immune function.

0550

IMPLEMENTATION OF HAEMOVIGILANCE SYSTEM FOCUSSING IN DONORS COLLECTION

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Introduction. Haemovigilance (HV) consists of detection, gathering and analysis of information regarding untoward and unexpected effects of blood transfusion. It covers and surveys all activities of blood transfusion chain from donors selection to recipients. The European Blood Directive 2002/98/EC requires a HV network in each Member State. In our country a national regulation has come into force related to the implementation of HV. Materials and *Methods.* Blood donation leaflets and medical questionnaire guidelines for donor deferral were elaborated. Personnel were instructed to inform the incidents related to blood collection using a standard preformatted form. Analysis of the data collected during 2005. Informatic System from Blood Bank was used to obtain statistical data from donations. *Results.* Global donations in our Blood Bank in 2005: 6844. Incidents related to donation: 72 (1.05%). Incidents in males 0.82% (44/5343), females 1.86% (28/1501). Moment of the incident: while donation 59,7% (43/72) and post-donation 54,16% (39/72). Type of incident: haematoma (2.77%), thrombophlebitis (1.39%), local infection (1.39%), neurological lesion (1.39%), nausea/vomiting (12.5%), clonic movements (4.17%), incontinence (1.39%), unconscious (30.5%), tetanic (1.39%), citrate reaction (1.39%), problems in venous access (4.17%), dizziness (51.9%). Room conditions: heat (38.9%), cold (1.39%), insufficiency (4.17%). Donors characteristic: low weight 6 (8,33%), previous reactions 4 (5.55%), anxiety 8 (11.11%), first time 16(22.22%), occasional donors 7(9.72%), common donors 39 (54.16%), auto-transfusion 9(12.5%).Graduation: immediate signs without life-treating risk and complete resolution 71 (98%) and immediate signs with life-treating risk 1 (1.38%). Imputability: possible 4(5.55%), suggestive 4(5.55%) and sure 64 (88.88%). *Conclusions.* Incidents related to donation are slightly higher in females. The most frequent event is the sickness related to donation, in most cases accompanied by signs without life-treating risk and complete resolution and with a sure relationship with donation.

Aplastic anemia

0551

A STUDY OF HLA AND KIR GENES IN PAROXYSMAL NOCTURNAL HAEMOGLOBINURIA PATIENTS

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Background. Paroxysmal Nocturnal Haemoglobinuria (PNH) is characterised by the occurrence of haemolytic anaemia, thrombophylia and cytopenia. The expansion of a stem cell bearing a somatic mutation in the phosphatidyl-inositol glycan-A (PIG-A) gene, which is involved in the biosynthesis of the glycosyl-phosphatidyl-inositol (GPI) anchor, characterises this very rare haematopoietic disorder. Murine KO models clearly indicate the inability of pig-a mutation to account alone for the clonal dominance of the GPI-defective clones and for the development of PNH. A number of data suggest the involvement of T-cell-dependent and NK-mediated mechanisms in the selection/expansion of the GPI-defective haematopoiesis in PNH patients. Moreover, the role of HLA and KIR genes in the regulation of adaptive and innate immune response has been established. *Aims and Methods.* In order to investigate the involvement of immune-dependent mechanisms in the pathogenesis of PNH we addressed the analysis of HLA and KIR gene distribution in 12 PNH patients and in 217 controls of the same ethnic origin by PCR-SSP typing. In addition, 15 patients affected by Aplastic Anaemia (AA), whose immune-mediated pathogenesis has been already demonstrated, were enrolled in the study. The statistical evaluation of data was performed by using Student's t test and Fisher two tailed exact test. *Results.* Our preliminary results demonstrate a significant increase of the HLA haplotype B*14, Cw*08 in PNH patients compared to healthy controls (36.3% vs 3.3%; $p<0.0005$) while a not significant increase of this haplotype was observed in our group of AA patients (13.3% vs 3.3%; $p= 0.087$). In addition, an increase of DRB1*13 was found in PNH (45.4% vs 20.1%; $p= 0.053$) but not in AA patients. KIR analysis showed a decreased expression of KIR-2DS3 (10% vs 32.7%; $p=0.110$) and an increased expression of 2DS2 (80% vs 52.1%; $p=0.077$) genes in PNH patients respect to controls. *Conclusions.* The critical involvement of HLA molecules in the regulation of the adaptive immune response and the relevance of KIR-repertoire for the functional effectiveness of NK and cytotoxic effectors have been largely recognised. In this context, our data support the hypothesis that complex immune-mediated mechanisms could underlie the dominance of the GPI-defective clones in PNH. The occurrence of ethnical differences as well as the number of patients enrolled in this study are expected to account for the apparent divergence with the increased frequency of DR2 observed in 21 Japanese and 16 American PNH patients.

0552

EXPRESSION PATTERNS OF GLYCOSYLPHOSPHATIDYLINOSITOL-ANCHORED PROTEINS (GPI-AP) THROUGHOUT THE DIFFERENT NORMAL BONE MARROW CELL MATURATION PATHWAYS: A FRAME OF REFERENCE FOR UNDERSTANDING PNH

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Introduction. Glycosylphosphatidylinositol-anchored proteins (GPI-AP) are a heterogeneous group of proteins deficiently expressed in patients with paroxysmal nocturnal hemoglobinuria (PNH). Despite the physiological and pathogenetic relevance of different GPI-AP in PNH patients, no study has been reported in which the exact patterns of expression of a large number of GPI-AP are quantitatively evaluated in normal bone marrow (BM) cells, classified according to their lineage and maturation stage. *Aim.*In the present study, we have quantitatively analyzed the expression of eleven different GPI-AP (CD14, CD16, CD24, CD48, CD52, CD58, CD59, CD66b, CD87, CD109 and CD157) during maturation of the neutrophil, monocytic, erythroid, lymphoid, basophil and plasmacytoid dendritic cell (pDC) lineages in normal BM as a frame of reference for the understanding of the abnormal patterns of expression of GPI-AP observed in the BM of PNH patients. *Material and Methods.* Ten normal BM samples from an iden-

tical number of healthy donors were analyzed by flow cytometry, using different 6-color stainings -depending on the specific cell lineage under study-, to analyze the expression of the above referred GPI-AP. *Results.* Our results show that expression of most GPI-AP varies during normal BM maturation, and different profiles were frequently observed depending on the cell lineage or the GPI-AP analyzed. Accordingly, the expression of CD55, CD58, CD24 and CD66b changed during the different stages of maturation of the neutrophil lineage (p<0.05), whereas CD59 remained stable. CD16 was expressed on myelocytes/metamyelocytes, increasing on the bands/mature neutrophils (p=0.001), while CD87 only became detectable on this latter stage. In a similar way, changes on the expression of CD55, CD58, CD59, CD109, CD14, CD87, CD157 and CD48 were observed during monocytic maturation. Different levels of expression of CD55, CD59 and CD58 were detected during the erythroid maturation. Maturation into basophils was associated with a higher expression of CD55 and a lower reactivity for CD59, both on this lineage and pDC. Finally, changes in the expression of CD55, CD52, CD24 and CD48 were observed along the B-cell maturation, whereas CD59 remains stable. *Conclusion.* Our study shows that the expression patterns of most GPI-AP vary along the different normal BM maturation pathways, and provides a detailed map GPI-AP expression during normal hematopoietic differentiation, which could serve as a basis for the identification and characterization of changes occurring in PNH.

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PREGNANCY-INDUCED PURE RED-CELL APLASIA, A DISTINCT SYNDROME

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Background. Pure red-cell aplasia (PRCA) is a rare hematologic disorder. Several conditions have been associated with the development of PRCA including malignancy, infections, thymoma, autoimmune disorders, and rarely pregnancy. Previously, we have described a patient who developed PRCA on three occasions, 2 triggered by pregnancy and 1 secondary to medroxyprogesterone. Here, we systematically review the information on all published cases of pregnancy-induced (P)-PRCA. *Aim.* To characterize the syndrome of P-PRCA. *Patient and Methods.* Published cases of PRCA induced by pregnancy were identified through MEDLINE (1966-July 2005; search terms: *pregnancy* and *red-cell aplasia, pure*) and references from journal articles, books and abstracts. We excluded patients who developed PRCA prior to pregnancy, or had other etiologies. This analysis focused on the patient characteristics; clinical aspects of PRCA; pregnancy features, infant characteristics, treatment and outcomes. *Results.* Ten patients with 13 P-PRCA episodes have been reported. *Patient characteristics.* Age ranged from 15 to 40 years. Gestational age at presentation varied from the first to the third trimester. No patient had other causes of PRCA. *P-PRCA.* Hemoglobin level at presentation ranged between 2.8 to 9 g/dL. Bone marrow evaluation showed marked erythroid hypoplasia with no abnormalities in the other cell lines. All patients received red blood cell transfusions, and 6 of them were treated with corticosteroids. Time to recovery of hemoglobin to a normal level ranged from 2 to 12 weeks post-partum, but was not described in three reports. *Pregnancies.* Four pregnancies ended with delivery via a Caesarean section, 1 at 30 weeks and 3 between 36-40 weeks of gestation. Three via vaginal delivery and 4 authors did not list the mode of delivery, all at full term. Two women underwent artificial abortions as treatment for PRCA. *Infants.* Fetal outcome included healthy infants in 8 cases and demise in 5. The cause of infant death was prematurity in 3, including 2 secondary to artificial abortions, and not specified full term still birth in 2. Infant blood values were normal in the 9 reported cases. *Follow-up.* Five subjects had subsequent pregnancies, 3 complicated by PRCA, 1 normal and 1 spontaneous abortion without PRCA. One woman developed PRCA secondary to the contraceptive medroxyprogesterone acetate 3 years after her first episode and 4 years before her second occurrence of P-PRCA. *Conclusions.* P-PRCA is a self limited syndrome with a high risk for relapse during subsequent pregnancies. It can be managed by blood transfusions. Progestins might cause PRCA in these women. Physicians should closely monitor women with a history of P-PRCA if they become pregnant again or receive hormones for contraception or other reasons.

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0554

IMMUNOSUPPRESSIVE THERAPY WITH ANTILYMPHOCYTE GLOBULIN, CYCLOSPORINE AND PREDNISOLONE IN THE TREATMENT OF APLASTIC ANEMIA A SINGLE CENTRE EXPERIENCE

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Immunosuppressive therapy offers a reasonable chance of cure in patients with aplastic anemia who do not have a HLA identical donor for allogeneic BMT. Between 1986-2005, 208 adult patients (age> 15 years) received immunosuppressive therapy with either Anti-lymphocyte globulin (ALG) or Anti thymocyte globulin (ATG). Equine ALG (Lymphoglobulin, Pasteur Mereiux) was given at the dose of 15 mg/kg/day for 5 days while equine ATG (ATGAM, Pharmacia Upjohn) was given at 40 mg/kg/day for 4 days. Following administration of ALG/ATG, oral prednisolone was started at the dose of 1 mg/kg/day for 10 days and in the absence of serum sickness, tapered over the next 7 days. From 1997, once steroids were tapered, Cyclosporine was started at 5 mg/kg/day in 2 divided doses for a period of 3-6 months and then tapered over 3-6 months depending upon the response. There were 145 males and 63 females with a median age of 36 years (range: 16-69). The median time from diagnosis to ALG was 3 months (range: 1-120). Ninetytwo patients (44%) fulfilled the criteria for Non-severe aplastic anemia (NSAA) while 86 (41%) had severe aplastic anemia (SAA) and 30 (15%) had very severe aplastic anemia (VSAA). ALG was given to 183 patients (88%) while ATG was given for 25 (12%). Cyclosporine was given to 101 patients (54.8%). Twenty four patients expired within one month of ALG administration related either to infection or bleeding while response was seen in 119 patients (57.2%). Among patients who showed a response, 51 had a complete response while 68 had a partial response. If the patients who expired within 1 month were excluded, the response rates were 64.6%. There was no difference in response between VSAA, SAA and NSAA (50%, 57%, 59.8%) but there was a significant difference between patients who received Cyclosporine versus those who did not (67.3% vs 41.1%; p=<0.001). On follow up, 8 patients evolved to paroxysmal nocturnal hemoglobinuria (PNH) while 1 patient transformed to a myelodysplastic syndrome (MDS). Four patients subsequently underwent allogeneic bone marrow transplantation and are alive and free of disease. At a median follow up of 26 months, the overall survival (OS) is 66% and the disease free survival (DFS) is 50.4%. In patients who were alive more than 1 month post ALG, the DFS was 57% with an overall survival of 69%. Immunosuppressive therapy with ALG/ATG offers a reasonable chance of response in patients with aplastic anemia with a sustained response in a number of these patients. Clonal evolution to PNH or MDS still remains a problem in patients receiving immunosuppressive therapy.

0555

SEVEN TURKISH PATIENTS WITH NIJMEGEN BREAKAGE SYNDROME: CLINICAL CHARACTERISTICS AND MUTATION ANALYSIS

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Background. Nijmegen breakage syndrome (NBS) (OMIM 251260) is a rare autosomal recessive disorder characterized by growth retardation, microcephaly, developmental delay, distinctive facial appearance, immunodeficiency and predisposition to malignancies. NBS is caused by mutations in the NBS1 gene which maps to chromosome 8q21. Mutations in the NBS1 gene were first found to be associated with this syndrome in 1998. The gene product, nibrin, is a novel protein which is the member of the hMre/hRad50 protein complex suggesting that the gene is involved in DNA double strand break repair. Most of the previously identified patients have belonged to Slavic populations, such as Poland, Czech Republic, and Ukraine. Almost all of patients from Slavic origin

were found to carry a homozygous five base pair deletion (657del5) in the 6th exon of this gene. Recently a Turkish patient with NBS have been reported and who was found to be homozygote for 657del5. *Aim.*We reported seven patients with NBS from three different families in Türkiye. Families of the three homozygotes, whom we identified, originated from a Central Anatolian city of Konya (2 families) and an Aegean city, Izmir. These families denied any relationship with Slavic populations. *Methods.* All probands in these families were phenotypically diagnosed as having NBS based on growth retardation, microcephaly, developmental delay and facial features in addition to lymphoreticular malignancies. Cytogenetic and immunological investigations also supported the diagnosis. *Results.* We identified three Turkish families in which probands were diagnosed as having NBS and found to be homozygote for 657del5. Evaluation of haplotypes created with help of three flanking microsatellite markers revealed that the 657del5 allele in three Turkish families had a single origine, which was identical with that found in the Slavic populations. *Conclusion.* This study demonstrated that NBS has not been very rarely diagnosed in Turkish population. Our detection of homozygotes in three unrelated families presenting with a malignancy implies that NBS is still underdiagnosed in Türkiye. 657del5 mutation in Turks shows the same origin described in Slavs. This result suggests the presence of population admixture in modern Türkiye.

0556

BONE MARROW EXPRESSION PROFILE OF RAS, P53, AND MDM2 GENES IN CYTOPENIC DISORDERS

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Ras, P53 and Mdm^2 genes are active regulators of cell growth, division, and death. These 3 genes form a cascade in which each gene is able to modify the transcription and expression of the other gene. In order to understand the mechanisms of cytopenias in different hematologic disorders, we investigated the expression of these genes in 16 patients with mono, bi or pancytopenia, excluding myelodysplastic syndromes (complementary data already published in Blood 2004 104: Abstract 3433). The expression of P21ras, P53, and Mdm^2 proteins was detected in bone marrow cytopins stained by APAAP (alkaline phosphatase anti-alkaline phosphatase procedure) using monoclonal antibodies Y13-219, PAb 1801 and 2A10, respectively. N-, K-, H-ras and p53 gene mutations were assessed by PCR-SSCP (polymerase chain reaction/single strand conformation polymorphism) to exclude the presence of mutations in these genes. The quantitative wild-type expression of p21ras, p53 and mdm^2 proteins in the cytopins (combination of number of cells and staining intensity) was compared to the values of expression of these proteins in 7 normal bone marrows. We found that patients with severe aplastic anemia (n=4), bone marrow hypoplasia (n=3), and toxic leucopenia (n=2) as diagnosis exhibited total loss of cytoplasmic expression or notorious hypoexpression of these proteins. Two cases of connective tissue disease (CTD) (1 undifferentiated connective tissue disease with positive pANCA; 1 systemic lupus erythemathosus) strongly overexpressed both P21ras and wild-type P53, while both cases of megaloblastic anemia (MA) aberrantly overexpressed wt-P53 protein. The other 3 patients with familiar leucopenia, hepatitis B thrombocytopenia and hypersplenism due to schistosomosis presented normal values of P21Ras, P53, and Mdm^2 proteins. We observed that P21ras, P53, and Mdm^2 proteins are downregulated in benign hypoplastic disorders of the bone marrow. Conversely, P53 and P21Ras proteins are upregulated in bone marrow disorders in which the cytopenia and the activation of these proteins are likely triggered by DNA lesions as well as subsequent apoptosis as part of the pathophysiology of the disease in CTD and MA. Hence, we suggest that P21Ras, P53, and Mdm^2 genes are tightly and actively modulated in benign cytopenic disorders of the bone marrow and play a pivotal role in the molecular mechanism of these diseases.

Anemia / Red blood cells II

0557

REDUCED INSULIN SECRETION IN NORMOGLYCEMIC PATIENTS WITH β-THALASSAEMIA MAJOR

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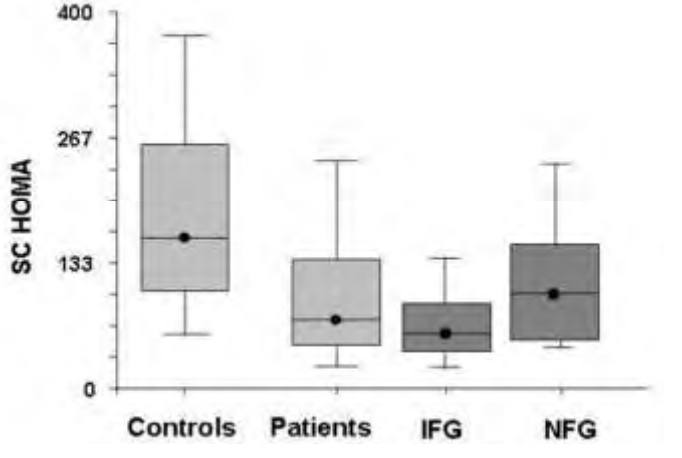
Background. Diabetes mellitus in patients with thalassaemia major is caused by hemosiderosis due to transfusional iron overload. However the exact mechanisms responsible for the progression from normoglycaemia to overt diabetes in these patients are still poorly understood. *Aims.* To assess insulin sensitivity and secretion in the fasting state in regularly transfused patients with β-thalassaemia major with normal glucose response during oral glucose tolerance test and estimate its possible relation to iron overload. *Methods*. We assessed fasting glucose, insulin and C-peptide levels from 24 patients with β-thalassaemia major and 18 healthy age- and body mass index- matched controls. Insulin sensitivity and insulin release index were calculated according to the HOMA model. The correlation to age, body mass index and serum ferritin was further analyzed. *Results.* Fasting glucose levels of patients were increased compared to controls (5.50±0.12 mmol/L vs. 4.67±0.13, mean±SEM, p<0.001).A decrease in b-cell secretion in the fasting state (estimated by SCHOMA) was observed in thalassaemic patients (SCHOMA 88.47±11.11 vs.184.29±23.72 in controls, p<0.001).Further intragroup analysis of patients to impaired (IFG) and normal (NFG) fasting glycaemia group , revealed an increased SCHOMA in NFG compared to IFG patients (110.63±17.63 vs. 66.31±10.88 respectively, p<0.05) but no difference was found regarding estimated insulin sensitivity (ISIHOMA) between the two groups. Plasma values of C-peptide correlated positively with ferritin (r=0.42, p=0.04) and SCHOMA (r=0.45, p=0.02) and negatively with ISIHOMA (r= '0.43, p=0.03). *Conclusions*. These results support the concept that an impairment of b-cell function, as reflected by a decreased insulin secretion index, already exists in _-thalassaemic patients with normoglycaemia before any changes in glucose tolerance can be detected in oral glucose tolerance tests.



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RECOMBINANT ERYTHROPOIETIN AS TREATMENT FOR THE HYPOREGENERATIVE ANEMIA OF HEMOLYTIC DISEASE OF THE NEWBORN

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Background. Intrauterine transfusions (IUTs), red cell transfusions (RCTs) and exchange transfusions (EXTs) are usually administered, in utero or during the first days after delivery, for the treatment of hemolytic disease of the newborn (HDN) due to Rh or ABO incompatibility. These transfusional practices induce a severe suppression of the erythropoiesis evidenced by reticulocytopenia, bone marrow erythroid hypoplasia, and inadequately low levels of serum erythropoietin (EPO). Spontaneous reactivation of erythropoiesis only occurs after 2-4 months of age. Consequently, a late hyporegenerative anemia gradually develops between 2nd and 6th weeks of life, and affected infants frequently

require RCTs. A few authors reported that recombinant EPO (rHuEPO) is useful for its treatment and prevention in neonates with Rh HDN who received IUTs or intrauterine EXTs. Furthermore, since the neonatal bone marrow is not able to sustain an adequate erythropoietic response throughout several weeks, this hyporegenerative anemia frequently develops in infants with severe ABO or Rh HDN who received no transfusion. No trial involving this population has been published. *Aims.* To evaluate the efficacy of rHuEPO for the treatment of HDN due to Rh, ABO or other antigens incompatibility, regardless of whether patients received or not IUTs, RCTs, or EXTs. *Methods.* After the first week of age, infants started treatment with epoetin α (Hemax®), 250 U/kg, subcutaneously, 3 times a week, when their hematocrit (Htc) dropped to levels requiring RCT, with a clear inadequate reticulocyte response. Patients were closely monitored throughout the following days and RCTs were administered, according to the criteria of the treating physician, if a further decrease of Htc occurred or if clinical signs and symptoms of anemia developed. The treatment was discontinued when the Htc reached normal levels. All patients were given iron (6 mg/kg/day) and folic acid (1-2 mg/day). *Results.* Twenty six infants were included (Rh HDN=19; ABO HDN=6; KpA HDN=1); 9 patients (ABO=6, Rh=3) had not been administered any IUTs, EXTs or RCTs, and 8/19 Rh HDN had received IUTs. Mean age at starting the treatment was 27.5±15.8 days (range: 8-65). Htc and reticulocytes count (Rtc) showed significant increases after 7 and 14 days of treatment (Table).

Table 1.

Patients	Hematocitic(%)			Reticulocye count (%)		
	Initial	Day 7	Day 14	Initial	Day 7	Day 14
All Patients	24.6	27.8[a]	30.0[a]	1.6	6.1[b]	6.1[c]
Rh+kpA Patients	24.0	27.5[b]	29.7[a]	1.5	6.3[b]	6.8[b]
ABO Patients	26.5	28.8[d]	31.0[b]	1.9	5.5[c]	4.2[d]

[a]p<0.001; [b]p<0.01; [c]p<0.005; [d]p<NS

No difference was observed between infants with Rh or ABO HDN. Comparison between patients with Rh HDN receiving or not IUTs showed no significant difference for: a) the Htc increase from day 0 to 7 (4.6±5.8% vs. 3.0±2.8%, respectively), or from day 0 to 14 of treatment (6.6±7.9% vs. 4.8±5.8%, respectively); b) the Rtc increase from day 0 to 7 (7.2±5.8% vs. 5.2±3.3%, respectively), or from day 0 to 14 of treatment (7.9±7.4% vs. 5.3±4.7%, respectively). Five neonates (19.2%) required one RCT at days 2, 3, 7, 16 and 24 of treatment (ABO=1, Rh with IUTs=3, Rh with no IUT=1). The rHuEPO was administered during 14 to 94 days (mean 37.8±17.7 days). No adverse effect was observed. *Conclusion.* The rHuEPO seems to be a safe and useful therapy for the late hyporegenerative anemia of HDN due to Rh or other incompatibilities, regardless the administration or not of IUTs or RCTs.

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RET-HE - RETICULOCYTE HEMOGLOBIN EQUIVALENT - IN PATIENTS WITH VITAMIN B12 AND/OR FOLATE DEFICIENCY

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Background. Ret-He is a new parameter obtained from the Sysmex XE-2100 counter, which measures the haemoglobin content of the reticulocytes. Its reference range is 30.2-36.7 pg. Low Ret-He values are observed in iron deficiency anemia (IDA). *Aim of the study*. Our aim was to study Ret-He levels in patients with vitamin B12 (B12) and/or folate (Fol) deficiency. *Patients and Methods.* Ret-He was studied in 121 patients with B12 / Fol deficiency (B12 <200pmol/l / red cell folate <600 nmol/L). There were 52 males and 69 females, with a median age of 74 years (18-99). Anemia was observed in 90% of the patients. The series includes the following patients: 55 with B12 deficiency; 24 with Fol deficiency; 14 cases with B12 and Fol deficiency; 25 with B12 deficiency associated with IDA and 3 patients with Fol deficiency and IDA. In the 94 cases with B12 deficiency, the levels of B12 were distributed as follows: B12 <100pmol/L n=42; B12 100-150pmol/L n=28 and B12 150-200pmol/L n=24. Hyperhomocysteinemia (hyperHcy), considered when serum homocysteine exceeded 17 micromol/L, was present in 84% of the cases with low B12.. In the 41 cases with Fol deficiency the median levels of red cell folate were 443 nmol/L, ranging from 263 to 565, with hyperHcy in 90% of them. *Results.* Ret-He was increased in patients with B12/Fol deficiency without IDA. The median values observed were: 36.6 pg in B12 deficiency; 35.6 pg in Fol deficiency and 39.7 pg in patients with B12+Fol deficiency, without significant differences between these 3 groups. Ret-He was significantly higher than in the reference group (p<0.001). In the group of patients that presented an associated IDA, Ret-He values were significantly decreased (median 24.9 pg; range 15-39) compared to patients with B12/Fol deficiency without IDA (p<001). *Conclusions.* Ret-He is increased in patients with vitamin B12/Fol deficiency. Moreover, a low Ret-He in patients with vitamin B12 or folate deficiency suggests the presence of an associated iron deficiency.

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DORSAL SURAL NERVE CONDUCTION STUDY IN VITAMIN B12 DEFICIENCY WITH MEGALOBLASTIC ANEMIA

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Backgrounds. Peripheral neuropathy is frequently observed in B12 deficiency. In spite of this, knowledge about peripheral neuropathy in B12 deficiency is little because the severity of clinical involvement of the central nervous system clearly outweighs signs and symptoms due to peripheral nervous system involvement. *Aims.* We primarily investigated peripheral neuropathy with dorsal sural nerve conduction study, which is a new method for detection of early peripheral neuropathy, in B12 deficiency with megaloblastic anemia. Also, as posterior column involvement is the most frequently reported and complicated neuropathy in B12 deficiency, tibial sensory evoked potentials (SEPs) were studied in all patients. *Methods.* Twenty-eight B12 deficiency patients (15 male, 13 female, mean age 65,8 years) with megaloblastic anemia and 18 age-and-sex matched controls were included. Dorsal sural nerve conduction studies, conventional motor/sensory nerve conduction studies and tibial SEP were performed. *Results.* Although dorsal sural sensory nerve action potentials (SNAPs) were not recorded in 15 (54%) of 28 patients, only 9 (32%) of them had polyneuropathy with conventional conduction studies. Furthermore, patients with dorsal sural SNAP had mean lower amplitude, mean longer latency and slower velocity response when compared to controls. Twenty patients (71%) were diagnosed with myelopathy with the combination of tibial SEP and neurological findings. Two patients whose dorsal sural SNAP were not recorded had normal tibial SEP responses; therefore, these patients were considered to have isolated peripheral neuropathy. *Summary/Conclusions*. As a result, we conclude that dorsal sural nerve conduction study is a reliable method for detection of early peripheral neuropathy in B12 deficiency. On the other hand, in concordance with previous studies, dorsal tract involvement is more common than neuropathy in B12 deficiency.

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ANTI-CARDIOLIPIN, ANTI-β2 GLYCOPROTEIN I, AND ANTI-PHOSPHATIDYLSERIN AUTO-ANTIBODIES AND THE RISK OF SICKLE CELL ANEMIA

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Background. Anti-cardiolipin (ACA), anti-β2 glycoprotein I (anti-β2GPI), and anti-phosphatidylserin antibodies (APS) were associated with thrombophilic disorders and hypercoagulable states, including deep venous thrombosis, recurrent pregnancy loss, and stroke. *Aims.* We determined the prevalence of these autoantibodies as risk factors for sickle cell anemia (SCA) among Bahraini patients and control subjects. *Patients/Methods.* This was a case-control study; study subjects comparing 78 SCA patients (mean age: 15.8±9.8 years) diagnosed with SCA according to hemoglobin profile, and 88 control subjects (mean age: 27.8 ±15.2 years) with no history oh hemoglobinopathies. ACA, aβ2GPI, and APS IgG and IgM levels were measured with ELISA; cut-off point was set as the mean value +3 SD of control subjects for each antibody. *Results.* Patients were matched to controls with respect to gender (p=0.073). ACA IgG (15.4% vs. 2.9%; p=0.01; OR = 6.09; CI = 1.31-28.71), and IgM (11.5% vs. 1.4%; p=0.02; OR = 8.87; CI = 1.09-71.92) were significantly higher among patients than in controls. Antiphosphatedylserin antibodies IgG (24.7% vs. 3.4%; p<0.001; OR = 9.27; CI = 2.61- 32.97), but not IgM (8.2% vs. 2.3%; p=0.142; OR = 3.85; CI = 0.75-19.69), were higher among patients than control subjects. In contrast, the prevalence of anti-β2GPI IgG (8.2% vs. 2.7%; p=0.166; OR = 3.22; CI = 0.63-16.53),

and IgM (4.2% vs. 1.6%; p=0.623; OR = 2.69; CI = 0.27-26.56) were comparable between SCA patients than in controls. *Summary/Conclusion.* ACA IgG and IgM and APS IgG are strongly associated with SCA among Bahraini patients, and their presence is potentially linked to hematological-coagulation abnormalities frequently seen in SCA.

0562

ERYTHROPOIETIN PRODUCED BY A HUMAN CELL LINE HAS ONLY TRACE LEVELS OF POTENTIALLY IMMUNOGENIC N-GLYCOLYLNEURAMINIC ACID RESIDUES

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Background. Recombinant erythropoietins are used extensively in the management of anaemia associated with chronic kidney disease or cancer. At present, all of these agents are produced in Chinese Hamster Ovary (CHO) cell lines. This leads to glycosylation patterns that differ greatly from that of endogenous human erythropoietin. This may be important as, in some bioactive substances (including growth factors), glycosylation patterns are thought to affect bioavailability, pharmacokinetics and functionality. Of particular interest is the presence of N-glycolylneuraminic acid (Neu5Gc) residues, as this substance is not produced naturally in humans. As a result, it has immunogenic potential and tests show that individuals have circulating antibodies to Neu5Gc. *Aims.* To produce an erythropoietin in a human cell line and characterize the structure, with a particular focus on Neu5Gc residues. *Methods.* An overview of the gene-activation technology (Shire Human Genetic Therapies, Inc.) used to produce erythropoietin (epoetin delta) is shown in Figure 1. The erythropoietin-producing cell line was created by transfection of a human cell line with DNA containing the appropriate targeting and gene-activating sequences. A variety of techniques have been used to characterize the resultant erythropoietin including: amino acid sequencing, peptide mapping with reverse-phase, high-performance liquid chromatography (HPLC)/mass spectrometry, oligosaccharide profiling and MALDI-TOF of released glycans. Sialic acid and Neu5Gc were quantified following labelling of the released glycans by reverse-phase HPLC analysis with fluorescence detection (limit of detection for Neu5Gc: 0.06 nmol/nmol epoetin delta). Neu5Gc content of recombinant erythropoietins was also assessed for comparison using the same technique. *Results.* Gene-activated erythropoietin, epoetin delta, can be produced as a highly pure and stable protein in a human cell line with a high potency towards reticulocyte production. It contains the full human primary amino acid sequence with no mutations or deletions. Epoetin delta contains trace levels of Neu5Gc residues (approximately 0.1% of total sialic acids) in comparison with recombinant erythropoietin produced in CHO cell lines (1-1.4%). Other differences in glycosylation pattern between epoetin delta and the CHO-derived recombinant erythropoietins are being identified. *Conclusions.* Epoetin delta, erythropoietin produced by a human cell line using gene activation technology, differs from CHO-derived recombinant erythropoietins in having far fewer Neu5Gc residues. Studies have shown that Neu5Gc containing substances are rejected in humans. The impact of highly immunogenic Neu5Gc residues on the profile of CHO-derived erythropoietins is not known, but studies are ongoing to evaluate whether the low content of Neu5Gc residues in epoetin delta provides any unique benefits.

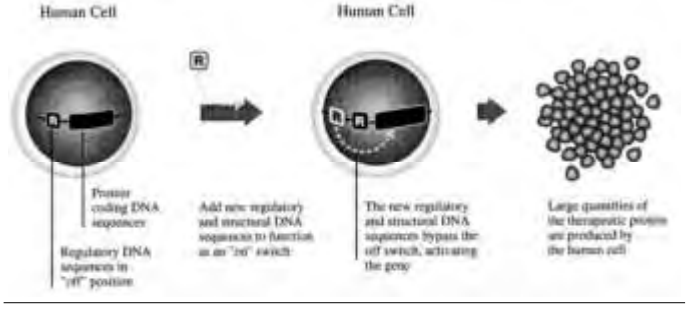

Figure 1. Technique used to produce epoetin delta.

0563

STUDY OF THE INNOVATIVE PARAMETERS RET-Y & RBC-Y IN PATIENTS WITH HYPOCHROMIC, MICROCYTIC ANEMIA

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Background. The RET-Y and RBC-Y (generated by Sysmex analyzer) are the mean value of the forward scatter light histogram within the reticulocyte and mature red cell population respectively, expressed in arbitrary units (AU). The RET-Y and RBC-Y are related to cell size and to cell content (mainly). *Aim.*The aim of this study was the assessment of the RET-Y and RBC-Y in patients with hypochromic, microcytic anemia and their correlation with soluble transferrin receptors (sTfR), sTfR/log ferritin ratio (sTfR-F index) and red blood cell indices (MCV, MCH, MCHC). *Methods.* We enrolled 116 patients with hypochromic, microcytic anemia (group A: iron deficiency n=39, group B: β thalassemia trait n=53, group C: O-Arab trait n=24) and 42 healthy individuals (group D). Blood counts were performed with Sysmex XT-2000i analyzer (in the reticulocyte channel). RET-Y and RBC-Y are provided by service data. Serum sTfR levels were determined with Dade Behring BN 100 nephelometer and ferritin levels were measured with Automated Chemiluminescence Systems ACS: 180_ SE Bayer. Statistical analysis: the data were expressed as the mean±SD. Group comparison tests were made by ANOVA. We applied t-Student test. We calculated Pearson correlations. Statistical significance was set at p<0.05.

Results.

Group A: RET-Y = 1521±164 AU*, RBC-Y = 1409±148 AU**
Group B: RET-Y = 1288±123 AU*, RBC-Y = 1208±137 AU**
Group C: RET-Y = 1644±139 AU*, RBC-Y = 1550±92 AU**
Group D: RET-Y = 1779±97 AU*, RBC-Y = 1655±86 AU**
(*), (**) the means for the four groups are significantly different (p<0.05).

The RET-Y parameter is significantly correlated with sTfR in groups A: r=-0.618, B: r=-0.358, C: r=-0.493 (p<0.023), with sTfR-F index in group A: r=-0.551 (p<0.001), with MCV in groups A: r=0.655, B: r=0.652, C: r=0.512, D: r=0.400 (p<0.01), with MCH in groups A: r=0.753, B: r=0.664, C: r=0.470, D: r=0.561 (p<0.02) and with MCHC in group A: r=0.727 (p<0.001). The RBC-Y parameter is significantly correlated with sTfR in groups A: r=-0.858, B: r=-0.326 (p<0.017), with sTfR-F index in group A: -0.828 (p<0.001), with MCV in groups A: r=0.848, B: r=0.644, C: r=0.580, D: r=0.461 (p<0.003), with MCH in groups A: r=0.935, B: r=0.635, C: r=0.584, D: r=0.590 (p<0.005) and with MCHC in group A: r=0.841 (p<0.001). *Conclusions.* Statistically different ranges for healthy individuals and patients with iron deficiency, β-thalassemia trait or O-Arab trait for RET-Y and RBC-Y indices exist. These two parameters are correlated positively with red blood indices MCV and MCH in all groups, while their values are related to cell size and to cell content (Hb). RET-Y is correlated negatively with sTfR in iron deficient and in enhanced erythropoiesis.

0564

LEBANESE G6PD DEFICIENCY: EVALUATION OF THE NEONATAL SCREENING AND PHENOTYPE DESCRIPTION

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Lebanese neonatal screening for G6PD deficiency is started in 1996 after a study showing an incidence of 12/000 and hemolytic anemia in 77.8% of those, mainly precipitated by beans. Molecular study in 36 of them showed Mediterranean form in 30/36. 95000 neonates were screened till January 2005 (20% of the Lebanese neonate). 503 have deficiency in G6PD: 1% of boys and 0.04% of girls. We conducted a study evaluating the screening and describing the phenotype of the Lebanese G6PD deficiency population. All the 503 patient family were contacted by phone. Only 123 could be reached. One refuses to cooperate. The 122 constituted the A population. From the 122, fifty were taken randomly. They constituted the B population with the 7 presenting hemolytic anemia in the A population. A questionnaire was done by phone with the A population and a more developed one with the B population. The same investigator has done all the questionnaires. There was 11 girls and 111 boys in the A population with a mean age of 4 years 3 months. 3/122 (2.46%) did not know about the result of the screening. 62/119 (52.12%) were informed of the deficiency orally by phone, 57/119 (47.82%) received the written list of 'substance to avoid' with the diagnosis. 7/122 (2 girls and 5 boys) present hemolytic anemia with 2 of

them needing transfusion. 3 of the 7 were the ones not knowing their deficiency. Mean age of hemolysis was 2 years 6 months. Beans ingestion precipitated hemolysis in 6 and viral infection in one. Beans ingestion was intentional in 3 cases. 20/122 (16.4%) lives near beans plantation and did not developed hemolysis. After announce of the deficiency 89/122 (73%) had consulted a physician. No one had consulted a hematologist. 20/122 (16.4%) did not know that G6PD deficiency could precipitate hemolysis. 80/122 (65.5%) ignores the transmission's modality of the disease. Age, sex, socio demography of the B population was similar to the A population. In the B population 31/57 (54.54%) have family story of G6PD deficiency. Twelve have done the screening because of the family story. 18/57 (31.5%) consider the disease as a serious one and only 31/57 (54.54%) consider this deficiency for life. 8/57(14.5%) have presented neonatal jaundice needing hospitalization. five of them (62.5%) received phototherapy. 42/57 (83%) knows that oxidative agent could precipitate hemolysis. From known oxidative agent only avoidance of beans showed significant reduces of hemolysis between exposed populations and none exposed. After hemolytic episode parents do not protect their children from other oxidative agent. Neonatal screening of G6PD deficiency reduces the risk of hemolytic anemia from 77.8% to 3.36%. 2.46% of the detected where not informed. Information is still lacking and need more reflection and follows up. Long list of substance to avoid do not necessarily guaranty avoidance of substance and compliance. Beans are the main substance to avoid.

0565

MEASURE OF RETICULOCYTE HAEMOGLOBINISATION BY WHOLE BLOOD HAEMATOLOGY ANALYSERS: CORRELATION OF CHR (BAYER ADVIA 120) WITH RET-HE (SYSMEX XE-2100)

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The haemoglobin content of the reticulocytes (CHr) has been used as a diagnostic tool in the diagnosis of anaemias and in monitoring erythropoiesis (1). The reticulocyte channel of Sysmex XE-2100 whole blood analyser provides a parameter defined as Ret-Y which corresponds to the mean value of the forward-scattered-light histogram within the reticulocyte population. By applying the regression plot Ret-He=5.5569exp 0.001Ret-Y, Ret-Y can be mathematically transformed into Ret-He an haemoglobin equivalent for reticulocytes, expressed in picograms (2). High correlation between Ret-He and the CHr parameter has been demonstrated, especially when monitoring iron deficiency anemia (3). The objective of this study was to establish the relationship between Ret-He and CHr parameters in reference Children Hospital of Athens Greece. A total of 300 random peripheral blood samples were analyzed. Blood samples comprised 200 healthy individuals, 30 patients with sphaerocytosis and 70 patients with haemoglobinopathies (mainly β-thalassaemia and Sickle Cell Disease/β thalassaemia). Blood counts were performed using two systems: Sysmex XE-2100 (Sysmex Corporation, Kobe, Japan) and Bayer Advia 120 (Bayer, Tarrytown, NY, USA). The within-run imprecision of Sysmex Xe-2100,concerning erythrocyte parameters, Hb, Hct, MCV and reticulocyte count was 0.8-1.4% and that of Bayer Advia 120 0.5-1%. The between-run imprecision of Sysmex Xe-2100 was 1.5-4% and of Bayer Advia 120 1.2-1.5%. The reference range for Ret-He was 28.8±5.4 pg (17.6 - 36.7 pg) and for CHr 27.9±4.3 pg (18.8 - 36.0 pg). Both values were normally distributed and showed a linear fit of the regression line. The regression equation calculated in this study was: Ret-He=1.16xCHr - 4.36, at 99% confidence limit (r=0.94, $p<0.00001$). Conclusively, the reticulocyte channel of Susmex Xe-2100 offers a new reticulocyte index Ret-He which shows excellent correlation with the equivalent parameter of Bayer Advia 120 Chr. Both parameters are equivalently functional in diagnosing different anaemias and in therapeutic monitoring of iron-restricted erythropoiesis.

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0566

EVALUATION OF MYOCARDIAL IRON DEPOSITION ASSESSED WITH M.R.I. IN YOUNG THALASSAEMIC PATIENTS RECEIVING ONE YEAR OF DEFERASIROX VERSUS DEFEROXAMINE

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Deferasirox (Exjade®) is a new once-daily, oral iron chelator, recently approved by FDA, while is awaiting EU regulatory approval. A multicenter clinical trial, recently published, indicated that daily administration of deferasirox at a dose of 20 mg/kg maintain liver iron concentration (LIC), whereas doses of 30 mg/kg achieve significant LIC reduction. Aim of this study was to evaluate the effectiveness of deferasirox in removing iron from the heart in comparison to deferoxamine, with the use of Magnetic Resonance Imaging (M.R.I.). In our center 11 young patients with β-thalassaemia major, aged 10 to 16.5 years with a mean age of 14.2±2.5 years, participated in a large multicenter, Phase III, comparative trial of deferasirox versus deferoxamine. Seven patients were randomized in the deferasirox group and 4 in the deferoxamine group. Doses were assigned according to baseline LIC assessed with percutaneous liver biopsy. In line with previous clinical management at our center, these patients were studied with myocardial MRI, as part of their routine monitoring, at the begging of the trial and one year after. MR images of heart were acquired during systolic phase, using electrocardiogram-triggered, flash 2D sequences, with 5 mm thickness and FOV 360-240mm. Region of interest (ROI) measurements were performed in the air and in the left ventricular myocardium. The natural logarithm of the signal intensity of the studied tissue to air ratio [ln (mean signal intensity of tissue / SD of air)], was calculated, with estimating normal values above 3.2. All patients completed one year of the study with no major adverse event. None of them was presented with any symptoms of cardiopathy, and heart echo, routinely performed according to the protocol design, was normal in every patient. All MRI's values were within normal range, something which can be attributed to the young of age. Mean heart MRI values at the begging of the study were 4.29 for the deferasirox and 4.3 for the deferoxamine group.

Table 1.

Agent	*Dose*	*n*	*MRI start*	*MRI end*	*p*
Deferasirox					
	10 mg/kg	1	4.21	3.6	
	20 mg/kg	5	4.37	4.25	0.17
	30 mg/kg	1	3.96	4.64	
	Total	7	4.29	4.21	0.32
Deferoxamine	35-50 mg/kg	4	4.30	4.42	0.28

One year after, heart MRI values were 4.21 and 4.41 respectively with no statistically significant difference. Of particular interest is the fact that one patient receiving deferasirox at a low dose of 10 mg/kg showed a significant reduction in MRI values (4.21 versus 3.60), whereas patient receiving high dose of 30 mg/kg managed to reduce myocardial iron deposit as indicated by the significant increase of MRI values (3.96 versus 4.64). Results are shown on the following Table. In conclusion, deferasirox at a daily dose of 20 mg/kg seems to be equivalent to deferoxamine doses of 40-50 mg/kg in maintaining myocardial iron concentrations. Similarly to liver, effect of deferasirox in removing myocardial iron is dose-depended Low dose of deferasirox (10 mg/kg) seems to be ineffective, whereas one patient receiving high dose showed an encouraging improvement in myocardial MRI values. Randomized, controlled studies are needed for safer conclusions.

0567

DIVERSE MOLECULAR DEFECTS ASSOCIATED WITH IDIOPATHIC ERYTHROCYTOSIS REFLECT THE HETEROGENEITY OF THIS DISORDER

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Background. Idiopathic erythrocytoses are a heterogeneous group of disorders characterised by an absolute increase in the red cell mass and associated with variable erythropoietin (Epo) levels. The diagnosis of diopathic erythrocytosis (IE) is one of exclusion in patients who do not

fulfil the criteria for the myeloproliferative disorder of polycythaemia vera (PV) and have no identified secondary causes such as a high affinity haemoglobin or Epo producing tumour. The recent discovery of the universal Janus Kinase (JAK2) mutation, V617F, associated primarily with MPD, now makes it possible to identify a clonal stem cell defect in those individuals who previously would have not fulfilled the criteria for PV and thus were included in the IE group. In the majority of IE cases the molecular defect is undefined. *Aims.* To identify the underlying genetic defects in IE individuals and establish if any of this group of patients would be positive for the PV associated V617F JAK2 mutation. *Methods.* DNA samples were prepared from more than 120 British and Irish erythrocytosis patients and PCR-direct sequencing of the following genes was performed: the cytoplasmic region of the Epo receptor (EpoR), all three exons of the von Hippel Lindau (VHL) protein and the catalytic domain of the prolyl hydroxylase PHD2. *Results.* Sequencing the EpoR identified a teenage boy with a truncation mutation, G6002A, which removed the terminal 70 amino acids from the receptor. This same mutation was first described in a Finnish skier but microsatellite analysis indicated that both mutations had arisen independently. Screening for the Chuvash VHL mutation, Arg200Trp, in the IE group detected 8 families from the Indian sub-continent who had members homozygous for this mutation. In addition, two Causcasian individuals both with erythrocytosis, D1 and E1, were heterozygous for the same mutation. Although E1 also possessed the G144R VHL mutation, D1 did not exhibit a second defect in the VHL gene but expressed the wild type allele. Most recently, a novel mutation, C950G, in PHD2 has been identified to cause erythrocytosis in 3 members of one family due to an aberration in the Epo negative feedback pathway. Finally, 64 IE individuals were screened by ARMS-PCR to indicate the prevalence of the V617F JAK2 mutation and only one individual was found to possess this mutation. *Summary.* Although mutations in the oxygen sensing pathway represent the major identified cause of IE so far, they account for only a minor proportion of the total number of patients with IE. Thus the molecular basis of a significant cohort of patients remains elusive but these individuals are a valuable resource that may provide insights into the mechanisms regulating red cell haemostasis and oxygen sensing.

0568

CHARACTERISATION OF BONE MARROW POSITIVE MITOGEN-STIMULATED DIRECT ANTIGLOBULIN TEST IN PATIENTS WITH REFRACTORY ANEMIA

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Background. Autoimmune phenomena, particularly directed against RBC, are described in Myelodisplastic syndromes (MDS). We already reported positive BM cultures in patients with refractory anemia (RA) and RA with ringed sideroblasts (RARS) by a new method named mitogen-stimulated-direct antiglobulin test (MS-DAT). *Aims.* We characterised the target BM cell of the MS-DAT positivity in MDS patients.

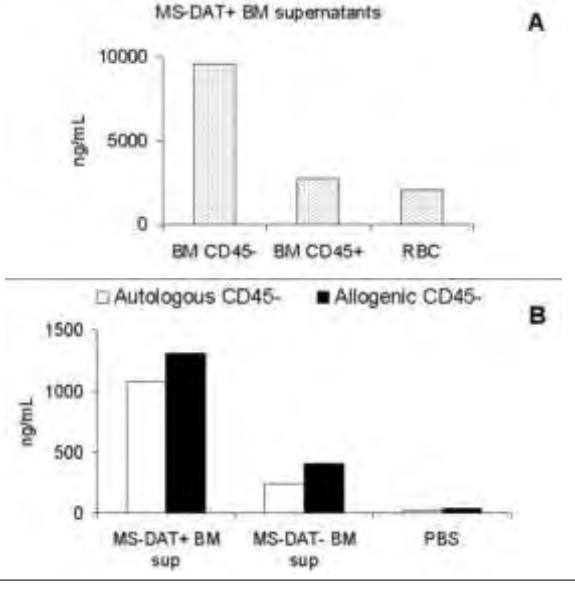

Figure 1.

Methods. MS-DAT was performed by stimulating BM cells with PMA and PHA and antibodies were detected in supernatants by competitive solid phase ELISA. BM cells were separated by magnetic beads in CD45+ (myeloid cells) and CD45- cells (erythroblasts) and supernatants of positive and negative cultures tested on both BM populations. *Results.* Eleven out of 23 patients showed positive MS-DAT in BM (cut off value 150 ng/mL±3SD), and positive patients had increased erythroblast counts and signs of hemolysis (i.e higher reticulocytes, indirect bilirubin, and LDH, and lower haptoglobin) compared with MS-DAT negative ones. *Results.* The supernatants of BM MS-DAT positive patients showed higher positivity on CD45- autologous BM cells, compared with CD45+ and RBC (Figure 1A). The reactivity was directed both against autologous and control allogenic erythroblasts (Figure 1B). MS-DAT negative BM supernatants had negligible reactivity with CD45- cells both from BM MS-DAT positive and negative patients. *Conclusion.* Our results show an autoimmune reactivity against erythroblasts in RA and RARS patients with peripheral signs of hemolysis.

0569

SERUM PRO-HEPCIDIN AND IRON STATUS IN THALASSAEMIA

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Background. Hepcidin is a antimicrobic-like hormone peptide synthesized in the liver. It seems to be a key regulator of iron homeostasis inhibiting intestinal iron absorption, recycling iron in the macrophages and mobilizing iron from hepatic stores. Hepcidin expression is induced by iron overload and inflammation and is suppressed by anaemia and hypoxia. Prohepcidin is a small plasma peptide believed to be a hepcidin precursor. Thalassaemia syndromes are a heterogeneous group of inherited anaemias resulting from reduced or absent synthesis of α- or β- globin chains of haemoglobin, where hepcidin is regulated by opposing factors such as ineffective erythropoiesis, anaemia and iron overload. In these conditions iron overload is mainly due to blood transfusions as weel as to increased iron absorption. *Aim.* To investigate serum pro-hepcidin in a cohort of Thalassaemia Major (TM) and Thalassaemia Intermedia (TI) patients and to evaluate a possible relationship with iron status. *Patients and Methods.* Thirty-three TM regularly transfused patients, twelve TI patients and twelve normal subjects were studied. TI patients had no or very few transfusions during their life, the last one being at least 5 years ago. Blood from TM was taken at least 48 hours after chelation therapy and just before blood transfusion. Iron status was evaluated by: serum ferritin, percentage of transferrin saturation and non transferrin bound iron (NTBI). Serum ferritin was determined by standard procedures; NTBI was assayed in serum by HPLC after nitrilotriacetic acid (NTA) chelation. Serum pro-hepcidin was measured by ELISA competitive binding assay (DRG,Germany). *Results.* Positive correlations were found between pro-hepcidin and ferritin (r=0.423, $p<0.01$), and between pro-hepcidin/ferritin ratio and NTBI (r=0.336, $p=0.05$) in TM patients. We report the results in Table 1.

Table 1.

	SF (ng/mL)	*NTBI (microM)*	*Pro-hepcidin (ng/mL)*	*Hb (g/dL)*
TM Pre-transfusion (n=43)	875±650°	0.86±1.23^	453±346°	9.0±0.5^
TI (n=12)	1183±777°	4.06±1.59*	548±395°	8.6±1.0^
Normal Subjects	170±80	-072±0.53	154±44	13.6±1.5

°p<0.0002 vs normal subjects; ^p<0.0008 vs normal subjects; °p<0.0001 vs TM normal subjects

Conclusions. In thalassaemia pro-hepcidin levels were increased for the degree of iron load and for the possible effect of concomitant minor infections. In thalassaemic syndromes where iron overload and anemia have opposing effect, the increased erythropoietic stress could influence hepcidin production. Understanding the mechanisms of iron homeostasis in patients with thalassaemia is of great significance in understanding the pathogenesis of iron load and planning novel treatments.

0570

THE ABSOLUTE RETICULOCYTE COUNTS AND IRON OVERLOAD CORRELATE WITH PULMONARY HYPERTENSION OBSERVED IN PATIENTS WITH SICKLE CELL/β THALASSEMIA

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Background. Echocardiographic studies have reported that approximately 30% of screened adult patients with sickle cell anemia have pulmonary hypertension (PH) defined as systolic pulmonary artery pressures of above or equal to 35 mm Hg or regurgitant jet velocity value (TRV) of above or equal to 2.5 m/sec. PH is increasingly observed in hemolytic anemias, including sickle cell disease and thalassemia in particular thalassemia intermedia. Brain natriuretic peptide (BNP) is released from the ventricles during pressure strain and its levels would correlate with severity of PH. *Aims.* The aim of this study was to evaluate the prevalence of PH in correlation with hemolytic findings and BNP levels in a cohort of patients with double heterozygous sickle cell trait and β-thalassemia (HbS/β-thal). *Methods.* We studied 52 patients (19 males and 33 females) with HbS/β-thal (thal 0: 35 pts and thal +: 17 pts) who were followed up regularly in the Thalassemia Center of Laikon Hospital. Their median age was 35 years (range: 21-62 years). All pts were evaluated for the presence of PH using Doppler echocardiography and then applying the modified Bernoulli equation (Pulmonary artery systolic pressure=4V(2) +right atrial pressure). Exclusion criteria of this study include: 1) evidence of left ventricular failure; 2) vaso-occlussive crisis during the last 15 days; 3) atrial fibrillation or vertricular tachycardia; 4) mitral value regurtitation (MVR) >2/4+ or mitral value stenosis; and 5) severe pericardial perfusion. In all patients we measured Hb, leucocyte and platelet counts, reticulocyte counts, serum lactate dehydrogenase (LDH), bilirubin, ferritin, creatinine, Hb F and BNP levels. Twenty-four (46%) patients were on hydroxyurea administration for a median time of 9±5.3 years. *Results.* Thirteen (25%) patients had PH, according to established criteria. All patients had mild symptoms, such as fatigue or dyspnea on slight exertion. The administration of hydroxyurea did not affect the presence of PH. Patients with PH had elevated values of reticulocyte counts and serum ferritin and a borderline increase of HbF compared with non PH patients. No other parameter was different between the two groups of patients (Table).

Table 1.

Parameter	*Patient with PH (n=13)*	*Patients without PH (n=39)*	*p-value*
Age (median; range)	41±9.4	36±13.9	
Gender (n)	7M/6F	12M/27F	
On hydroxyurea (n)	6 (46.1%)	18 (46.1%)	0.92
Hb (g/dL, mean±SD)	9.1±1.4	8.8±1.5	0.31
Retics (x1000/mm^3) (mean±SD)	230±86	175±65	0.01
LDH (U/L; mean±SD)	772.5±359.7	782.7±348.7	0.46
Bilirubin (mg/dL, mean±SD)	2.3±1.7	2.4±1.2	0.45
Creatinine (mg/dL; mean±SD)	0.7±0.1	0.8±0.3	0.10
Ferritin (μg/L; mean±SD)	1192.6±1142.4	449.1±694.8	0.02
BNP (pg/mL; mean±SD)	202.0±226.2	310.8±656.6	0.18
HbF (%)	16.9±8.3	13.1±9.8	0.09

Conclusions. The results of this ongoing study have shown that the frequency of PH in our cohort of HbS/β-thal patients is similar with that observed in patients with sickle cell disease. The correlation between PH with reticulocyte counts and ferritin suggests that the degree of hemolysis and iron overload may be implicated in the pathogenesis of PH in HbS/β-thal. There was no correlation between serum BNP or LDH and the presence of PH; however, this may reflect the number of patients available in the present study.

0571

CHARACTERISATION OF A DOUBLE MUTATION, THE NOVEL PRO92HIS AND PREVIOUSLY DESCRIBED GLU255-, OF NADH-CYTOCHROME B5 REDUCTASE ASSOCIATED WITH APPARENT TYPE I RCM

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Background. The clinical disorder of recessive congenital methaemoglobinaemia (RCM) is characterised by a deficiency of NADH-cytochrome b5 reductase (cb5r). There are two phenotypic forms of this disorder, type I and type II and although both types exhibit cyanosis from birth type II disease is accompanied by severe neurological defects. The cb5r enzyme consists of two distant sub-domains that comprise the FAD- and NADH-binding lobes, linked together by a hinge region. It participates in several pathways including the reduction of methaemoglobin, cholesterol biosynthesis and fatty acid metabolism. More than 40 different mutations have been described for cb5r that result in both forms of RCM and with the development of a heterologous expression system it is possible to characterise novel mutations in terms of enzymatic function and protein stability. *Aims.* To identify the molecular defect causing apparent type I RCM in a young girl and to characterise the identified cb5r variants for protein stability and enzymatic activity using a heterologous expression system. *Patient and Methods.* A DNA sample was prepared from an apparent type I RCM patient and PCR-direct sequencing of the DIA1 gene, which encodes cb5r, was performed. The resultant variants were generated using a bacterial expression system. For comparison the wild-type domain and the previously described RCM variant, Pro95His, were also prepared. All proteins were purified to homogeneity and characterised for enzyme activity and thermostability. *Results.* Sequencing detected a heterozygous deletion of GAG, bases 27,100 to 27,102 (NCBI accession number NT_011520) in exon 9 resulting in loss of Glu255. In addition, a homozygous C to A change at base 16,076 (C16,076A) in exon 4 predicting an amino acid change of proline to histidine at codon 92. Uniquely, in this case one allele carries a double mutation. Previously, a change of proline to histidine at codon 95 was described for an individual with type II RCM. Investigation of both the Pro92His and Pro95His mutations indicated they impacted moderately on the enzymatic activity of cb5r without dramatic changes towards the NADH substrate or affecting the redox potential of the FAD prosthetic group. The thermostability of both Pro92His and Pro95His variants was greatly decreased, indicated by a reduction of T50 by 11o and 9oC respectively compared to wild type enzyme. In contrast, the Pro92His and Glu255- double mutant exhibited substantially decreased enzyme activity, lower affinity towards NADH and reduced thermostability. *Summary*. Characterising the Pro92His and Glu255 variants, described in a type I RCM patient, indicated that the Pro93His mutation did not dramatically affect the function of cb5r but greatly decreased the thermostability of the protein. In contrast, the double mutation affected both the catalytic activity and the stability of the protein. The previously described Pro95His mutation also exhibited decreased protein stability but when present in combination with the Tyr42Ter mutation, resulted in type II RCM. Consequently, the pathophysiology of RCM appears to be influenced by the residual activity of the individual cb5r variants and the heterologous expression system provides a valuable tool in delineating between both types of RCM.

0572

COST ASSESSMENT OF B THALASSEMIA MAJOR: THE ITHACA STUDY

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Background. People with severe anemia like β-thalassemia major require blood transfusions as life-long therapy. Regular blood transfusions can however cause iron overload that may damage vital organs, particularly the liver, the heart and the endocrine glands. Iron chelation therapy is essential to prevent end-organ damage and improve survival. Currently, the most available drug is Deferoxamine (DFO), parenterally administered by continuous infusion. Deferiprone (L1) is an oral iron chelator but it is indicated for patients who have contra-indications for DFO therapy, or for patients in whom DFO was proven to be ineffective. So far, only little is known about the cost of care of subjects with thalassemia undergoing iron chelation treatment and about their satisfaction and quality of life. Aims to investigate the costs, compliance,

quality of life and satisfaction of patients who are currently undergoing iron chelation therapy. *Methods.* The Italian Thalassemia Cost and Outcomes Assessment (ITHACA) is a naturalistic, multicenter, retrospective study involving patients with β-thalassemia major of any age and who are on iron chelation therapy for at least 3 years, sequentially enrolled at 8 Italian Thalassemia Care Centers. Information on socio-demographic, clinical, resource utilization, quality of life and treatment satisfaction was collected through a battery of questionnaires. This analysis pertains on estimating the cost of care for thalassemic patients from an Italian society perspective. Data is expressed in € 2005. *Results.* 135 patients were enrolled (median age=27.8 years, ranging from 2.7 to 48.5 years), 68 (50.4%) were male and were followed up for 11.7 months on average. At the time of enrolment 109 (80.7%) patients had at least one complication related to thalassemia (mean number of complications per patient: 2.9). 51.5% of patients were treated with DFO administered by continuous infusion, 32.1% were treated with oral L1, 16.4% were treated with combined chelation therapy (DFO+L1). Direct medical and non medical costs were evaluated. Overall, the average cost was 1162.3 €/patient/month; the major cost driver was chelation therapy including drugs and administration costs, and it represented 53.1% of total costs, followed by transfusions (35.6%), surgical interventions (3.6%), laboratory and instrumental tests carried out in outpatient or day hospital setting and medical visits (3.3%), concomitant medications (1.7%). Direct non-medical costs, such as transportation, represent 2.9% of total costs. On average, the daily cost of infusion with Desferal was estimated to approximately 22 € excluding pump and consumables. *Conclusions.* This is the first study to assess the cost of care of patients with β-thalassemia major in Italy. Our findings show that transfusion and iron chelation procedures together account for the great part of short term (1 year) health care cost. These results can be considered conservative since consumables, nursing, home care and long-term complication costs are not included. Studies are needed to estimate indirect and long-term cost of thalassemia major in order to obtain a more accurate and broader picture.

0573

A FAMILY WITH A MILD PHENOTYPE DESPITE MULTIPLE MUTATIONS IN THE α- AND β-GLOBIN GENE CLUSTER

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Background. Hemoglobinopathies refer to a diverse group of disorders caused by an abnormal structure of the hemoglobin molecule. Thalassemias are hereditary disorders characterized by defective production of hemoglobin. Hemocytometry and hemoglobin variant analysis are the laboratory tests to study these hemoglobin disorders. In routine diagnostics, molecular analysis is not structurally applied. We describe a family with a generally mild anemic phenotype despite multiple mutations in the α- and β-globin gene cluster. *Aims.* The aim of this study was to clarify the aberrant hemocytometry and hemoglobin variant results of the proposita and her family by means of molecular analysis. *Methods.* Hemocytometry results were obtained using the Cell-Dyn 4000 (Abbott). Hemoglobin variant analysis was performed by cation exchange HPLC (Biorad). DNA sequence analysis was performed using the ABI 310 genetic Analyzer (Applied Biosystems). *Results.* The proposita (female, age 20) was referred to our laboratory upon suspicion of thalassemia (Hb 9 mmol/L, MCV 72 fl, MCH 1.62 fmol, erythrocytes 5.56x 10^{12}/L). She complained of fatigue but had no other clinical symptoms. Quantification of hemoglobin variants showed 11.3% HbA1, 4.4% HbA2, 58.9% HbC, and 23.0% HbF relative to total hemoglobin. On one allele of the patient we detected an HbC mutation (codon 6 GAG to AAG) in cis with the nonfunctional γA and γG 16C>G promoter sequence variation (unpublished results). At the second allele three molecular alterations were found in the β-globin gene cluster: the 90C>T β-globin promoter mutation that is known to cause β+-thalassemia and two novel mutations in the promoter of the γA gene (271C>T) and γG gene (403_392delCTTTAA). Further analysis of the patient's α-globin gene cluster revealed also the presence of a heterozygous α-thalassemia (3.7 deletion). DNA analysis of four family members revealed, in addition, several other sequence deviations in the γ-globin genes. Three common mutations were detected in the γG gene promoter: 222_226del (A)AGC(A), 309A>G, and 369C>G, whereas one novel mutation was detected in the γA gene promoter: 499T>A. The first of these mutations is known to lower γG globin expression, the second is associated with increased HbF levels in normal healthy adults, the third is not associated with increased HbF levels in normal adults, and the functionality of the latter is unknown at present. *Conclusions.* In the here presented family a total of ten different mutations were found in the globin genes: one in the α-globin locus, two in the β-globin genes, and seven in the γ-globin genes. In spite of this, the proposita and all family members displayed a mild clinical phenotype. It is possible that, ultimately, this beneficial mild phenotype results from the interplay between the various identified genetic variants which function as phenotypic modifiers. This case shows that molecular analysis subsequent to biochemical analysis can be beneficial, but that the need for such analysis should always be considered in relation to the clinical practice.

0574

GENOMIC INSTABILITY IN SICKLE CELL DISEASE PATIENTS USING HYDROXYREA ASSESSED BY ALKALINE SINGLE-CELL GEL ELECTROPHORESIS ASSAY

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Hydroxyurea (HU) is considered and antineoplastic drug, which also plays an important role in treatment of sickle cell disease patients (SCD). Short-term HU toxicities primarily include transient myelosuppression, but long-term HU risks have not been defined. The mutagenic and carcinogenic potential of HU is not established, although HU has been associated with an increased risk of leukemia in some patients with myeloproliferative disorders. In the present study we analyzed the presence of DNA damage in patients with SCD treated with HU, in peripheral blood lymphocytes, using alkaline single-cell gel electrophoresis assay. We analyzed 36 patients with sickle cell disease (16 males and 20 females), aged 2-59 years (mean 25,75±14,5), received oral HU median dose of 26,5 mg/Kg/day, for a period of 0.6- 11,8 years (mean 5,01). The control group was composed of 23 healthy individuals (10 males and 13 females), aged 4-52 years (mean 26,61±7,20). The results revealed that damage index in SCD patients was significantly higher than in controls (p=0.0014). This study indicates a possible genotoxicity of the HU, although further works are necessary to evaluate its mutagenicity.

0575

DISTINCT PHENOTYPIC EXPRESSION IN PATIENTS WITH THE HYPERUNSTABLE ALPHA-CHAIN VARIANTS HB TAYBEE (ALPHA1CD38/39DELACC; THR>0) AND HB HERAKLION (ALPHA1CD36/37DELCCC; PRO>0)

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α-thalassemia mutations are of the commonest in humans. Amongst more than 80 α-thalassemia mutations, more common are deletions, partially or completely removing the α-globin gene cluster (16p13.3, ζ2-ψζ1-ψα2-ψα1-ψ2-α1-θ). Point mutations within either α-globin gene (non-deletion mutations) are less common. Non-deletion mutations usually reduce α-globin synthesis by impeding RNA processing or translation, but some cause post-translational hyper-instability of the abnormal polypeptide, mimicking α-thalassemia through an overall reduction of α-globin synthesis. Interaction of non-deletion α-thalassemia determinants usually causes Hb H disease, a chronic moderate anemia in which excess β-globin chains form Hb H (β-4). We observed 6 Greek cases with an atypical β-like thalassemia phenotype: chronic moderate anemia without abnormal hemoglobin fractions. DNA analysis characterized common α+-thalassemia mutations in-trans to in-frame 3bp deletions in the α1-globin gene: 4 cases (2 unrelated children and 2 adult siblings) had codon36/37,delCCC (Hb Heraklion), and a further 2 unrelated adults had codon38/39,delACC (Hb Taybee), which represents the first observation of Hb Taybee in Greece. Three Hb Heraklion cases with the non-deletion IVS-1 donor splice-site mutation (αHphα) in trans had Hb levels 70-95 g/L; the fourth Hb Heraklion case and both Hb Taybee cases had α+-thalassemia 3.7kb deletion in trans, maintaining Hb levels around 105 g/L. All cases had slight to moderate hemolysis (bilirubin 3 to 7-fold normal) and increased erythroid marrow activity indicating dyserythropoiesis (serum erythropoietin and soluble transferrin receptor levels 3 to 5-fold normal). Furthermore parameters indicating the status of oxidant-antioxidant balance showed moderate impairment of the glutathione system and an increased rate of lipid peroxidation, reflected by increased levels of malonyldialdehyde. Absence of detectable abnormal hemoglobin fractions (including Hb H) in such cas-

es confounds diagnosis based on hematology alone, and definitive diagnosis is only achieved through DNA analysis. To date Hb Heraklion has been observed in a single Greek case and Hb Taybee in sporadic Israeli-Arab cases. There is minimal experience for the management of such atypical cases, and our previous experience indicates that it is probably insufficient to monitor clinical status in patients with hemoglobinopathies based on hemoglobin levels alone.[1]

Reference

1. Stamoulakatou *et al.,* BCMD, 32: 118-123, 2004.

0576

EPOETIN DELTA, ERYTHROPOIETIN PRODUCED BY A HUMAN CELL LINE, IS EFFECTIVE IN THE TREATMENT OF RENAL ANAEMIA

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Background. Several recombinant erythropoietins are currently available for the treatment of anaemia associated with chronic renal failure and cancer. All of these agents are produced in Chinese Hamster Ovary cell lines and as a result have glycosylation patterns that differ from endogenous erythropoietin. Epoetin delta (Dynepo™, Shire) is an erythropoietin produced in a human cell line through gene activation. *Aims.* To assess the efficacy and safety of different doses of epoetin delta in patients with anaemia and chronic renal failure requiring haemodialysis. *Methods.* In this multicentre, double-blind study, haemodialysis patients with anaemia (haemoglobin < 10.0 g/dL) who had not previously received an epoetin were randomized to receive epoetin delta (15, 50, 150 or 300 IU/kg) or epoetin alfa (50 IU/kg) three times a week. In the initial *correction* phase, patients received the allotted dose until 'correction success' was reached (two consecutive weekly haemoglobin measures ≥ 11.5 g/dL or one measurement ≥ 13 g/dL). Patients achieving correction success then entered a maintenance phase, during which the dose was titrated to maintain haemoglobin levels at ≥ 10.5 g/dL. The maximum duration of treatment was 12 weeks. Maintenance success was defined as haemoglobin ≥ 10.5 g/dL at week 12. Total success was defined as achievement of both correction success and maintenance success. Data from the groups assigned the two highest doses of epoetin delta were pooled and compared with results from the lowest dose group. *Results.* In total 78 patients were randomized and 75 received treatment (epoetin delta 15, 50, 150 and 300 IU/kg: 21, 14, 13 and 13 patients, respectively; epoetin alfa 50 IU/kg: 14 patients). Baseline haemoglobin levels were similar in the pooled epoetin delta and epoetin alfa groups (8.66±0.94 and 8.57±0.82 mg/dL). The proportion of patients achieving total success was higher in the pooled highest dose epoetin delta group (150 and 300 IU/kg) compared with the lowest dose (15 IU/kg) group (55.6% vs. 4.5%; p=0.0002). Analysis of dose trend across the epoetin delta groups showed a significant trend for an increase in total success and correction success with increasing doses (15, 50, 150, 300 IU/kg: total success, 4.5, 21.4, 50.0, 61.5%, respectively, p=0.0001 for trend; correction success, 9.1, 21.4, 57.1, 61.5%, respectively, p=0.0002 for trend). There were no significant differences in success rates between epoetin delta 50 IU/kg and epoetin alfa 50 IU/kg. The incidence of treatment-emergent adverse events was similar in the epoetin delta and epoetin alfa groups. Adverse events thought to be possibly related to epoetin delta occurred in 11.5% of patients and most were mild or moderate in severity. There was no evidence of dose-related adverse events. *Conclusions.* Epoetin delta is effective in increasing haemoglobin levels in patients with haemoglobin < 10g/dL as a result of chronic renal failure, and shows at least similar efficacy to epoetin alfa at an equivalent dose. Safety profiles were similar for the two agents. No patient receiving epoetin delta developed anti-erythropoietin antibodies.

Cytogenetics and Molecular Cytogenetics

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ONCOGENIC DEREGULATION OF HOXA GENES BY CHROMOSOMAL REARRANGEMENTS IN T-CELL LYMPHOBLASTIC LEUKEMIAS (T-ALL)

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T-cell acute lymphoblastic leukemias (T-ALL) are highly malignant tumors which derive from partially differentiated T-cell progenitors. We recently reported the identification of a new recurrent chromosomal rearrangement in human T-ALL, targeting the major homeobox gene cluster HOXA and the T-cell receptor genes locus TCRB (Soulier *et al.*, Blood 2005). This rearrangement was found in four patients out of a series of 92 T-ALL and corresponded to inv(7)(p15q34) and t(7;7)(p15;q35), in 3 and 1 cases, respectively. The 4 HOXA breakpoints were analysed at the molecular level by Southern blot in the 4 cases, and cloning of the two derivative breakpoints in two cases. The breakpoints clustered within a 2.6 kb region in the HOXA locus. In order to analyse the molecular consequences of this rearrangement, the expression of the 11 HOXA genes was analysed on micro-array data and by specific RQ-PCR. We found that the whole HOXA gene cluster expression was deregulated in the rearranged cases, on both side of the breakpoint cluster region, compared to other T-ALLs. Mechanisms of this deregulation remains elusive. Two additional groups of T-ALL demonstrated a global HOXA cluster deregulation, namely the CALM-AF10 and the MLL-rearranged T-ALL cases. These results strongly suggested that the deregulation of HOXA genes is oncogenic in T-ALL. Global gene expression analysis and unsupervised hierchical classification in the 92 cases T-ALL series demonstrated that the TCRB-HOXA associated cases clusterised in an homogeneous subgroup, which shared common expression profile with the TLX1/HOX11 and TLX3/HOXL2 associated subgroups. This suggested use of common biological oncogenic pathways in these homeobox genes associated T-ALL. Like other T-ALL, these cases frequently demonstrated NOTCH1 gene activationg mutations and CDKN2A/p16/ARF deletions, consistent with multi-events oncogenesis. We then analysed expression of two alternative transcripts, HOXA9b and HOXA10b, considering the clusterization of the breakpoints between the HOXA9 and the HOXA10 genes in the TCRB-HOXA translocated cases. Interestingly a massive expression of the HOXA10b transcript was demonstrated, whereas no significant expression was detected in the CALM-AF10 and MLL-associated T-ALL cases, or in other T-ALL cases. The HOXA10b transcript has been detected during early embryogenesis in mice and in leukemic cell lines. It encodes a short HOXA10B protein which retains the homeobox domain of the regular HOXA10 protein (HOXA10A) but lacks the N-terminal regulation domain. We found no expression of HOXA10b during T-cell differentiation by analysing normal human thymus samples, showing that its expression in T-cell leukemic cells was ectopic. Considering the specific expression of the HOXA10b transcript in the TCRB-HOXA cases, we are currently analysing the phenotypic consequences of the HOXA10b homeobox gene overexpression in mouse models using retroviral transduction of bone marrow progenitors.

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CORRELATION BETWEEN CHROMOSOMAL ABNORMALITIES AND IMMUNOHISTOCHEMICAL PROFILE IN DIFFUSE LARGE-B CELL LYMPHOMAS REVEALS DISTINCT LYMPHOMAGENESIS PATHWAYS WITH CLINICOPATHOLOGIC SIGNIFICANCE AND PROGNOSTIC VALUE

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Background. DLBCL constitutes a heterogeneous group. The genetic and molecular mechanisms underlying their diverse clinical presentations and outcomes have been partially clarified by the recent application of DNA microarrays and tissue arrays technologies. Cytogenetic studies of DLBCL also revealed a broad spectrum of clonal genetic abnormalities and complex karyotypes, including, chromosomal translocations, deletions, duplications and other complex alterations. However the potential clinicopathological relevance of these alterations is still poorly defined. *Patients and Methods.* 101 previously untreated patients

diagnosed with *de novo* DLBCL at our hospital between 1987 and 2003 were selected (median age = 59 years, 50 males, IPI 0-1 : 32% ; 2-3 : 46%; 4-5 : 22%). The inclusion criteria were the availability of appropriate paraffin embedded-tissues and a karyotypic analysis using R-banding method. Hierarchical clustering analysis based on immunostaining with a large panel of antibodies (including cell-cycle control, apoptosis, immune response and B-cell differentiation markers) was performed and correlated with recurrent cytogenetic abnormalities and outcome. The germinal center B-cell like (GCB) and the non-GCB phenotypes were defined using CD10, BCL6 and MUM1 immunostaining. *Results.* Among the 101 studied patients, 10 karyotypes were considered as normal. The most frequent numerical genetic abnormalities were monosomy 15 (19%), trisomy 3 (15%), 7 (33%) 11 (17%) 12 (26%) 18 (17%) and X(20%). The most frequent structural abnormalities involved 1p (31%), 1q (33%), 2p (21%), 3p (16%), 3q (45,3%), 4q (21%), 5q (16%), 6q (38%), 7q (19%), 8q (15%), 9p13 (26%), 11q (15%), 14q32 (49%), and 18q (35%). The t(14;18), t(3;14) and t(8;14) were observed in 21%, 20% and 2% of cases respectively. The GCB phenotype was observed in 46% of cases and is significantly related to t(14;18) (36%), trisomy 12 (36%), and 18q21 (45%) or 2p (31%) rearrangements. The non-GCB phenotype was observed in 54% of case and correlated with 3p (23%) and 3q (57%) rearrangements. DLBCL with t(14;18) are preferentially CD10+ (72%), BCL2+ (68%) and MUM1 negative (56%). By contrast, DLBCL with t(3;14) were more often p53+ (41%), MUM1+ (94%), usually expressed the anti-apoptotic galectin-3 molecule (70%) but were BCL2 negative (88%). Using an unsupervised hierarchical clustering approach based on the expression of a large panel of antibodies, 82% of cases could be properly reclassified only by considering the presence of a t(14;18) or of a t(3;14), indicating clearly 2 distinct cells of origin. Finally, p53 protein expression correlated with 17p and 3q27 rearrangements. Clonal genetic abnormalities with a significant unfavourable prognosis impact were the 17p, 3p, 8q24 and 9p13 rearrangements. A scoring system, including all unfavourable genetic abnormalities was strongly predictive of the outcome, independently of the GCB/ non-GCB phenotype and was confirmed in an independent series of 87 DLBCL. In addition to these clonal genetic abnormalities, BCL2, CD5 and p53 expressions were associated to a poor clinical outcome. *Conclusion.* This study demonstrates correlation between chromosomal abnormalities and immunohistochemical profile in DLBCL, and reveals distinct lymphomagenesis pathways with clinicopathologic significance and prognostic value. These results contribute to a molecular database which could allow the identification of new relevant genes involved in lymphomagenesis.

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PDGFRB FUSES TO TPM3 IN THE T(1;5)(Q23;Q33) OF CHRONIC EOSINOPHILIC LEUKEMIA AND TO NDE1 IN THE T(5;16)(Q33;P13) OF CHRONIC MYELOMONOCYTIC LEUKAEMIA

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Background. Ph-negative chronic myeloid leukemia, namely atypical chronic myeloid leukemia and chronic myelomonocytic leukemia (CMML) with bone marrow and/or peripheral blood eosinophilia are associated with PDGFRB/5q33 translocations. The 3' region of PDGFRB encoding the kinase domain fuses with the 5' region of a partner gene encoding an oligomerization domain, which determines the constitutive activation of PDGFRB tyrosine kinase. To date, ten different PDGFRB gene partners have been identified and several PDGFRB translocations with undefined gene partners have been also observed. *Aim.* Identification of new PDGFRB partners in patients with 5q33 translocations. *Methods.* Patient 1, a 21-year-old man with chronic eosinophilic leukemia showed a 46,XY,t(1;5)(q23;q33) karyotype in 28/29 metaphases. The patient underwent α-IFN and oncocarbide treatment for ten years and switched to imatinib after identification of a PDGFRB rearrangement. Patient 2, a 36-year-old woman with Noonan syndrome and exon 3 PTPN11 missense germline mutation, was admitted because of CMML. Cytogenetics showed the following karyotype: 46,XX,t(5;16)(q55;p13)[11]/46,XX[3]. After assessing PDGFRB involvement imatinib mesylate therapy was administered and haematological, cytogenetic, and FISH remission was achieved after six months. FISH with two cosmids for PDGFRB/5q33 (9-4 and 4-1) was performed in both patients. DNA clones for the 1q23 band in patient 1, and for 16p13, in patient 2, were applied to narrow breakpoints and to select candidate partners. RT-PCR with gene-specific primers were performed to amplify TPM3/PDGFRB and NDE1/PDGFRB fusions from patients 1 and 2, respectively. Amplicons were sequenced for confirmation. Functional assays were perfomed on the NDE1/PDGFRB fusion by testing transduced Ba/F3 cells for IL-3 independent growth. Sensitivity of the NDE1/PDGFRB fusion protein to imatinib mesylate was assessed. *Results.* Cosmids for PDGFRB gave a red/green fusion signal on normal 5 and a split signal with cosmid 9-4 retained at der(5) and cosmid 4-1 translocated to der(1) (patient 1) or der(16) (patient 2). In patient 1, the 1q23 breakpoint fell within clone RP11-205M9. In patient 2, DNA clone CTD-2303E13 NDE1/16p13 and cosmids 27/29 for the 3'MYH11/16p13 were present on normal 16 and on der(5) while cosmids 14/18 for the 5'MYH11/16p13 and clone RP11-8H13 MRP/16p13 gave one signal on normal 16. In patient 1, RT-PCR for TPM3/PDGFRB amplified a chimeric transcript joining TPM3 exon 7 to PDGFRB exon 11. In the second case, RT-PCR showed fusion between exon 5 of NDE1 and exon 11 of PDGFRB. Ba/F3 cells transduced with NDE1/PDGFRB fusion were transformed to IL-3 independent growth. The NDE1/PDGFRB fusion protein was shown to be sensitive to imatinib. *Conclusion.* We identified TPM3/1q23 and NDE1/16p13 as two new PDGFRB/5q33 partners in patients with CEL and CMML, respectively. Interestingly, the TPM3 gene is already known for its involvement in the t(1;2)(q23;p23)/TPM3-ALK of anaplastic large cell lymphomas and for its recombination with NTRK1 in papillary thyroid carcinomas, while NDE1 haploinsufficiency has been associated with the onset of a MDS-like syndrome in mice.*Acknowledgements.* DNA clones were kindly provided by Dr. M Rocchi, University of Bari, Italy.

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A DNA-BASED RQ-PCR SCREENING ASSAY FOR RUNX1 COPY NUMBER CHANGES IN CHILDHOOD B CELL PRECURSOR ACUTE LYMPHOBLASTIC LEUKEMIA

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Background. An increased RUNX1 copy number is a common finding in BCP-ALL. It usually identifies additional copies of chromosome 21, which in turn is a typical feature of hyperdiploid cases. In a much smaller proportion of cases, however, it results from rearrangements that specifically multiply the region 21q22 (intra-chromosomal RUNX1 multiplication; ICRM). This distinct genetic marker, also known as AML1 amplification, designates a specific form of BCP-ALL with a pronounced risk of relapse. Noteworthy, less than 100 such cases have been reported so far worldwide. The apparent rarity results in part also from the fact that at present an ICRM can only be detected with fluorescence *in situ* hybridization (FISH) and a systematic FISH screening is only conducted in very few treatment trials. *Aim and Methods.* To overcome this diagnostic obstacle, we developed a DNA-based real-time polymerase chain reaction (RQ-PCR) screening assay. It is based on the comparative quantification of three regions within RUNX1 at 21q22, PRSS7 at 21q21.1 (as an intra-chromosomal control) and BBS1 at 11q13.2 (as an inter-chromosomal control). The assay was set up and evaluated with DNA from cases with two (normal controls), three (Down syndrome patients) and four (hyperdiploid ALL) chromosomes 21 and put to test on samples from 13 Austrian cases with a previously FISH-verified ICRM. The number of additional RUNX1 copies in these samples was determined to range from 4 to approximately 8. *Results.* Screening of 221 BCP ALL samples from the German BFM-ALL trial identified altogether 88 cases (40%) with an increased RUNX1 copy number. Two of them had a constitutional trisomy 21, five (2,3%) an ICRM, 65 (29%) were hyperdiploid and 16 (7%) were ETV6/RUNX1-positive and had an extra copy of chromosome 21. *Conclusion.* The respective PCR results were in good accordance with those suggested by DNA-index, cytogenetic or FISH analyses. They prove that such a DNA-based screening technique can reliably identify and delineate RUNX1 overrepresentations in different genetic BCP ALL sub-forms, such as those with an ICRM, a hyperdiploid or pseudotetraploid karyotype as well as those ETV6/RUNX+ ones with secondary changes. Since this approach is extremely well suited for the fast and efficient retro- and prospective analyses of a large number of cases we foresee that it will become the preferred method for the identification of such cases in childhood ALL treatment trials.

0581

META-ANALYSIS OF 966 B-CELL NEOPLASMS WITH 8Q24 ABERRATIONS IDENTIFIES DISTINCT CYTOGENETIC ABERRATION PATTERNS OF BURKITT AND NON-BURKITT LYMPHOMAS

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Background. Burkitt lymphoma (BL) is cytogenetically characterized by a translocation juxtaposing the MYC locus on band 8q24 next to the IGH locus on 14q32 or one of the light chain loci on 2p12 and 22q11. However, translocations affecting the MYC locus are not exclusive for BL but also occur in other B-cell neoplasias. *Aims.* Based on published karyotypes derived from conventional cytogenetic analyses, we intended to define the typical cytogenetic signature of Burkitt lymphoma and to delimit this signature from other B-cell lymphomas (B-NHL) with 8q24 translocations. A typical cytogenetic signature could be used as adjunct for clinical diagnosis, and may point towards loci targeted by disease-specific genetic events. *Methods.* We performed a meta-analysis of karyotypes from 966 B-cell neoplasms from the Mitelman with 8q24 breakpoint in the main clone using software from the Progenetix project. 461 cases were diagnosed as Burkitt lymphoma or leukemia. The remaining cases consisted (in decreasing number) of ALL (NOS), DLBCL, B-NHL (NOS), myeloma, FCL and other B-NHL entities. *Results.* 440 BL cases lacked a translocation involving 3q26-q27 or 18q21. Of those typical BL, 258 had chromosomal imbalances (average 1.3 imbalances, median 1 imbalance per case). The 346 non-BL B-NHL with cytogenetic translocations indicating a MYC/IG fusion showed a greater overall chromosomal instability (average 3.7 imbalances, median 3 imbalances). In BL, recurring gains involved 1q21q32 (21%), chromosome 7 (7%) and chromosome 12 (5%). Losses were uncommon, with a maximum of 4.5% on 17p. Other regions affected by chromosomal aberrations in B-NHL like 3q, 6q, 13q and 18q were rarely imbalanced in BL. No differences were observed for lymphomatous and leukemic variants of BL. Gains on 1q and 12 were nearly exclusive in BL, with co-occurrence in only one case. In contrast, the 21 BL cases with additional 3q26-7 or 18q21 break exhibited typical B-NHL aberrations, such as 6q26 loss (15%) or 18q gain (10%) but no gain on 1q. The non-BL B-NHL cases with cytogenetic translocations indicating a MYC/IG fusion displayed a heterogeneous pattern of imbalances. As in BL, the most common gains involved 1q22q31 (24.9%), 7 (19.4%) and 12 (10%). However, chromosomes 19p (10.1%), 3 (9.8%), 9q (9.4%) and others (21, 11q, 5, 15) had frequent gains, too. Recurring losses involved 6q21 (11.9%) and 13q (11.9%). In contrast to the BL subset, limitation to single chromosomal imbalances was much less common (37% for 0 or 1 imbalance in non-BL B-NHL vs. 70% in BL; $p<0.001$ for distribution). *Summary.* Burkitt lymphomas with cytogenetic translocations indicating a MYC/IG fusion contain only few, usually single genomic imbalances. The low complexity of BL underscores the etiologic importance of the IG/MYC fusion in this disease. The mutually exclusive pattern of imbalances may point to alternative genomic events co-operating with IG/MYC translocations in BL. BL cases with additional *B-NHL* abnormalities may be part of a distinct disease group. Non-BL B-NHL with 8q24 translocations display a heterogeneous pattern and larger number of chromosomal imbalances. Our analysis exemplifies the importance of large data collections for determining relevant cytogenetic aberration patterns.

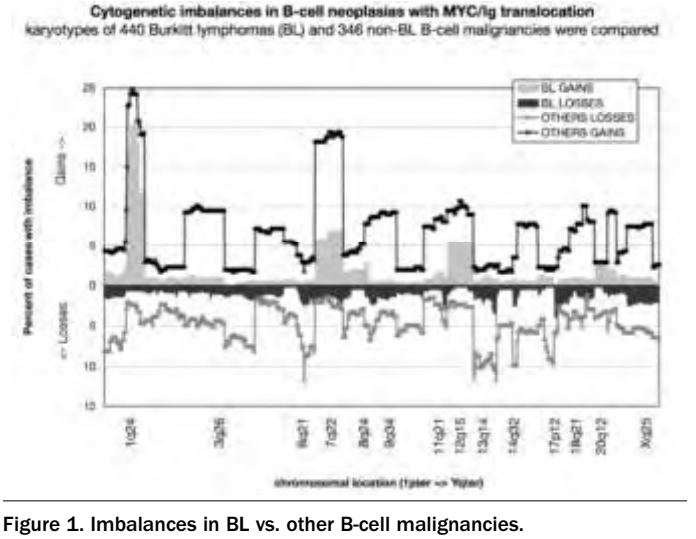

Figure 1. Imbalances in BL vs. other B-cell malignancies.

0582

MOLECULAR CHARACTERIZATION OF DISTINCT HOT SPOT REGIONS ON CHROMOSOME 7Q IN MYELOID LEUKEMIAS

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Background. Loss of whole chromosome 7 (-7) or deletion of the long arm (7q-) are recurring chromosome abnormalities in myeloid leukemias. In recent years, several groups initiated the molecular characterization of the deletion and translocation breakpoints. Based on these results a commonly deleted segment (CDS) of approximately 2 Mb in size was identified in chromosomal band 7q22 flanked by the microsatellite markers D7S1503 and D7S1841. Recently, we mapped the translocation breakpoint of a t(3;7)(p13;q22) within this genomic segment and identified a novel gene (MLL5, mixed lineage leukemia 5) that represents a candidate gene for chromosome 7 associated leukemias. With respect to deletions affecting the distal part of chromosome 7q a 4 to 5 Mb sized CDS was defined encompassing chromosomal bands 7q35 to q36. However, several other CDS and translocation breakpoints on 7q have been described so far, suggesting the existence of more than one disease-related gene. *Aims.* To identify and characterize translocation and deletion breakpoints in a large series of myeloid leukemias with chromosome 7q aberrations using FISH and array CGH. Once, novel hot spot regions are identified, transcriptional map(s) are constructed with the intention to identify candidate genes. *Methods.* FISH with a physical map of well defined YAC (yeast artificial chromosome) clones representing the long arm of chromosome 7 was performed on a series of 105 myeloid leukemias including acute myeloid leukemia (AML), myelodysplastic syndrome (MDS) and myeloproliferative disorders (MPD). In addition, selected patients were analysed by array CGH and results were confirmed by hybridisation of the corresponding DNA clones. Transcriptional map(s) were constructed by the use of public databases. *Results.* While most of the deletions were large and encompassed the previously published CDS, we identified a distinct 2 Mb sized genomic segment in the proximal part of 7q22 that was defined by five patients. This segment contains several candidate genes including the putative tumor-suppressor genes CUTL1 and RASA4. Interestingly, this CDS is located close to multiple miR-sites, which usually indicate common fragile sites in the human genome. In chromosomal bands 7q35-q36 we localized the breakpoint of an unbalanced translocation from a patient with secondary AML between the markers D7S1925 and D7S1395. This region was recently characterized as a common fragile site in the human genome, named FRA7I. Furthermore, the translocation breakpoint t(3;7)(p13;q35) of a patient with therapy-related AML was cloned into a 100 kb sized genomic segment that is located centromeric the CNTNAP2-gene close to the proximal border of the CDS. *Conclusions.* Our data further indicate the remarkable heterogeneity of deletion and translocation breakpoints on 7q and revealed several hot spot regions that may serve as important starting points for the identification of pathogenetically relevant genes.

0583

SERIAL ANALYSIS OF CHROMOSOME ABERRATIONS IN MULTIPLE MYELOMA: HIGH PREVALENCE OF STRUCTURAL ABNORMALITY OF CHROMOSOME 1 DURING DISEASE PROGRESSION

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Background. Two major genetic subtypes of multiple myeloma (MM) have been proposed: the hyperdiploid subtype characterized by multiple trisomies and low prevalence of del(13q), and the non-hyperdiploid subtype characterized by IgH translocations and del(13q). Primary IgH translocations have been considered as initiating events in the pathogenesis of MM. The role of del(13q) and other chromosomal abnormalities in disease progression have not been clarified. *Aim.* To investigated the evolution of chromosomal abnormalities during disease progression. *Methods.* Analysis of the cytogenetic abnormalities of serial bone marrow samples from MM patients that entered in the clinical cytogenetic database at Erasmus MC. *Results.* Seventy-five serial samples obtained from 36 patients at diagnosis (26 samples) and during progression of the disease (45 samples) were included in this study. The mean interval between the samples was 26 months (range 6-77). Samples without clonal abnormalities were considered as failures. Using conventional cytogenetic banding techniques, 13/36 (36%) initial and 19/39 (49%) follow-up samples had an abnormal karyotype. The origin of respectively 19 and 37

marker chromosomes could not be identified. Of the remaining 32 samples with abnormal karyotype 15 were hyperdiploid and 17 non-hyperdiploid , respectively. Serial studies showed an increased number of chromosomal abnormalities during disease progression. The mean number of aberrations increased from 11 (range 1-38) to 18 (range 2-36). Trisomies of chromosomes 5, 9, 11, 15 and 19 were the most common numerical abnormalities. Monosomy of chromosome 13 was identified in 3/13 initial and 7/19 follow-up samples with an abnormal karyotype. Aberrations of chromosome 1 were the most common structural abnormality (25/162) (15%) and were detected in 2/13 initial and 10/19 follow-up samples. Both the short and long arms of chromosome 1 were involved and no-specific locus was predominantly affected. The rearrangement of chromosome 1 consisted in the majority of unbalanced translocations and resulted in gains of 1p/1q. Aberrations of chromosomes 3 and 8 were second in frequency (6%) and were detected in 1/13 initial and 9/19 follow-up samples. FISH analysis was performed in 56 samples and showed an abnormality in 15/22 (68%) initial and 25/34 (74%) follow-up samples with probes specific for RB-1 (13q14) and D13S319 (13q14.3) loci and for the centromere regions of chromosome 9 and 11. Del(13q) was observed in 10/22 (45%) initial and 14/34 (41%) follow-up samples. *Summary.* Cytogenetic abnormalities in multiple myeloma are not random. Disease progression is correlated with increasing complexity of cytogenetic karyotype, which consist mainly of structural aberrations acquired during later stages of the disease. Aberrations of chromosome 1 are common in multiple myeloma. In particular, unbalanced translocations of 1p/1q have been delineated as genetic event associated with progressive disease and unfavourable prognosis. Del(13q) is not associated with disease progression.

0584

THE APOC1 GENE LOCATED ON 19Q13.2 IS RECURRENTLY GAINED AND OVEREXPRESSED IN ACUTE MYELOID LEUKEMIA

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Background. Trisomy 19, as a sole anomaly, is a characteristic finding in *de novo* myeloid malignancies. However, the gain of chromosome 19 mainly appears with other chromosomal abnormalities, in the accelarate or blastic phase of chromic myeloid leukemias and in acute megakaryoblastic leukemias (AMKL). By simultaneous arrayCGH and expression profiling analysis, we have previously reported a list of candidate oncogenes that are gained and overexpressed in AMKL cell lines within a common chromosome 19 amplified region.[1] This list pointed to six genes that were recurrently involved, apparently being APOC1 the target of the amplicon. APOC1 has been confirmed in other series of expression array experiments as one of the recurrently overexpresed genes in Acute Megakaryoblastic leukemia.[2,3] *Aims.* 1.-To characterize the genomic status of APOC1 on myeloid malignancies. 2.-To confirm the expression of APOC1 on AMKL *Methods.* We have designed a molecular assay to detect copy number changes of APOC1 in DNA leukemic samples based in the multiplex ligation-dependent probe amplification (MLPA) technique.[4] We have examined a total of 14 primary AML samples with normal and complex kayotypes (F.A.B subtypes M0, M1, M2, M3, M4 and M5) and 10 AML cell lines. The APOC1 protein expression was studied by immunohistochemistry in the AMKL. *Results.* The MLPA analysis reveals extra copies of the APOC1 gene in 28% of the primary cases. Surprisingly, two out of the 9 samples with normal karyotype at diagnosis showed the same aberration, demostrating that this event is also present in non-AMKL leukemias. On the cell lines a good correlation with the previously described APOC1 amplification was confirmed. This event was present only on the cell lines derived from AMKL patients The APOC1 protein expression was confirmed by immunohistochemistry in the 6 AMKL cell lines with chromosome 19 gain, and was not detected in the 3 AMKL cell lines without this chromosomal gain. We are currently studying the status of APOC1 expression in 65 primary samples by immunohistochemistry. *Summary/Conclusions*. Using a MLPA technique we have found extra copies of the APOC1 gene in 28% of the primary cases. APOC1 is an apolipoprotein, extensively studied on lipid metabolism, whose function in leukemogenesis is not well understood. We have confirmed the presence of this protein in all the cell lines with gain of chromosome 19. Further studies are needed to estimate the frequency of this event on AML and to established the role of APOC1 overexpression in leukemogeneis.

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0585

CHROMOSOMAL ABERRATIONS ARE DETECTED IN 80% OF CLL PATIENTS BY METAPHASE CYTOGENETICS: A STUDY OF 132 CLL CASES WITH CORRELATION TO FISH, IGVH STATUS, AND CD38 EXPRESSION

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Chronic lymphocytic leukemia (CLL) is a heterogenous disease from a clinical as well as from a genetic point of view. Compared to fluorescence *in situ* hybridization (FISH) conventional metaphase cytogenetics plays only a minor prognostic role in chronic lymphocytic leukemia (CLL) so far due to technical problems resulting from limited proliferation of CLL cells *in vitro*. Here we present a simple method for *in vitro* stimulation of CLL cells which overcomes this limitation. CLL cells were induced to metaphase generation by culture in normal growth media with the addition of the immunostimulatory oligonucleotide DSP30 plus interleukin 2. In our unselected patient population 125/132 cases could be successfully stimulated for metaphase generation. 101/125 cases showed chromosomal aberrations. The aberration rate is comparable to the rate detected by interphase FISH, which was performed in parallel. Conventional cytogenetics detected additional aberrations in 47 patients compared to FISH analysis. A complex aberrant karyotype, defined as ≥ 3 aberrations, was detected in 30/125 patients compared to only one such case as defined by FISH. Samples with 17p deletions in FISH had a complex aberrant karyotype in 83% of cases. Conventional cytogenetics frequently detected balanced and unbalanced translocations. A significant correlation of the poor prognosis unmutated IgVH status with unbalanced translocations and of the likewise poor prognosis CD38 expression to balanced translocations and complex aberrant karyotype was found. We demonstrate that FISH analysis underestimates the complexity of chromosomal aberrations in CLL. Therefore, conventional cytogenetics may define subgroups of patients with high risk of progression.

0586

T(4;11) PATIENTS REVISED: T(4;11) PATIENTS CARRY TWO MLL FUSION ALLELES

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The chromosomal translocation t(4;11) is the most frequent chromosomal aberration of the human MLL gene, associated with infant and early childhood acute lymphoblastic leukemia (ALL) or therapy-related acute leukemia. The disease phenotype is correlated with poor Prednison-response and outcome. Thus, leukemia patients carrying t(4;11) translocations are treated according to high-risk acute leukemia therapy regimen. Compared to other MLL translocations, no cellular or animal model system is currently available which mimics the human t(4;11) translocation phenotype. It has been reported that the conditional expression of an MLL•AF4 fusion protein causes cell cycle arrest instead of proliferation. Therefore, t(4;11) translocations seem to belong to another disease mechanism that cannot be explained by the current knowledge about other MLL fusions where the der(11) is responsible for the oncogenic character. The AF4 fusion partner is a potent oncoprotein in mammalian cells. The oncogenic properties were located in the N-terminal portion of AF4, the same portion fused to the MLL in the AF4•MLL fusion protein. The latter fusion protein was able to growth-transform mammalian cells equally well as H-ras, a well known oncoprotein. However, this finding is in contrast to the fact that only about 80% of all t(4;11) patients seem to encode both reciprocal fusion genes (MLL•AF4+/AF4•MLL+ patients), while for 20% of these patients, only the presence of the MLL•AF4 fusion gene (MLL•AF4+/AF4•MLL-

patients) could be successfully identified either by RT-PCR or by direct genomic PCR. This controversial data provoked us to investigate 'MLL•AF4+/AF4•MLL-' leukemia patients in more detail by using a LDI-PCR based method. This allows to identify and characterize chromosomal aberrations of the human MLL gene in an unbiased fashion. 13 individual MLL•AF4+/AF4•MLL- leukemia patients out of 76 t(4;11) leukemia patients were indentified (63 were MLL•AF4+/AF4•MLL+ leukemia patients). The 13 MLL•AF4+/AF4•MLL- leukemia patients were analyzed for the presence of rearranged genomic MLL sequences. 10 patients displayed a complex rearrangement between chromosome 4 and 11 (and sometimes a third chromosome) that involved at least the MLL, the AF4 and a third partner. Funded by grant 2002.061.1 from the Wilhelm Sander Foundation to R.M., T.K. and T.D.

0587

FISH-MLL ABNORMALITIES IN PATIENTS WITH ACUTE MYELOBLASTIC LEUKEMIA AND ASSOCIATION WITH FLT3 AND MLL INTERNAL DUPLICATION

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Background. The MLL gene on chromosome 11q23 is frequently involved in haematological malignances. It is possible to subdivide the MLL abnormalities in two groups: 1) rearrangements, usually as translocations or insertions, and partial tandem duplication (PTD); 2) amplification of the 11q23 region, leading to the presence of multiple copies of the MLL gene, located either intrachromosomally, as hsr and iso11q, or extrachromosomally in dmin and numerical abnormalities of chromosome 11. MLL/PTD is the in-frame fusion of a duplicated portion of the MLL gene. Internal tandem duplication (ITD) or mutations have been demonstrated as a activating mechanism also in another oncogene involved in AML, FLT3 gene, which encodes for a tyrosine kinase receptor widely expressed in hemopoietic lineage. The FLT3/ITD is observed in approximately 20% of unselected *de novo* adult AMLs, with a higher frequency around 30-40% reported for patients with normal cytogenetics. It is associated with poor prognosis in most series. It has been reported that FLT3/ITD is more common in patients with MLL/PTD than in cases with MLL translocations. Recently, a role for coduplication of MLL and FLT3 genes has been suggested in AML as possible marker of a common genotoxic stress. *Aim*. We investigated the incidence of MLL abnormalities in 207 patients with *de novo* acute myeloid leukemia, diagnosed following FAB criteria and treated according to the GIMEMA protocols. We used conventional cytogenetics and fluorescent *in situ* hybridization (FISH) analysis with a MLL probe. The patients were also tested for the presence of an internal duplication of the MLL and FLT3 gene and for the FLT3 D835 mutation. *Methods and Results.* Cytogenetic analysis on bone marrow was successful in 175 cases and showed abnormalities of chromosome 11 in 12 patients (6.9%). FISH analysis performed with MLL Dual-Color probe (Vysis) was available in 194 cases and demonstrated the MLL involvement in 25 cases (12.9%). Ten patients were rearranged (5.2%); 15 cases showed overrepresentation of MLL gene without evidence of rearrangement (7.7%). FLT3/ITD or D835 mutation were observed in 27.4% and MLL/PTD in 5.3% of the patients. FLT3 abnormalities were present in 20% (5/25) whereas MLL/PTD was observed in 18.7% (3/16) of the cases with involvement of MLL at FISH analysis. *Conclusions.* The FISH investigation of MLL contributes to the identification of multiple copies of the gene in marker chromosomes, rings, double minutes, hsr. The presence of MLL amplification is not rare in *de novo* AML and the FISH analysis allows to improve the characterization of MLL involvement when compared with conventional cytogenetics. The incidence of FLT3 alterations is similar in MLL abnormal patients (20%) when compared to the whole AML population (27.4%); on the contrary, MLL/PTD is confirmed to be more frequent in patients with abnormalities of chromosome 11 (18.7% vs 5.3% of unselected AML). The rate of MLL/PTD was superior in FLT3 positive (7.7%) than in FLT3 negative patients (4.4%). In this study the coduplication of FLT3 and MLL/PTD had a low incidence around 2.3% in all cases and did not correlate with cytogenetic MLL abnormalities.

0588

A NEW CRYPTIC MOLECULAR LESION UNDERLIES 6P CHANGES IN SECONDARY ACUTE MYELOID LEUKEMIA/MYELODYSPLASTIC SYNDROME (AML/MDS)

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Background. Secondary AML/MDS are frequently associated with complex karyotypes involving chromosomes 3, 5, 7, 11, 12, 17, 18, and 21. Specific genetic pathways are related to physical and/or chemical toxics, such as -5/5q- to alkylating agents or 11q23 and 11p15 changes to topoisomerase II inhibitors. 6p aberrations are cytogenetically heterogeneous and often belong to complex karyotypes with del(5)(q)/-5 and/or del(7)(q)/-7. *Aim.* Molecular characterization of 6p rearrangements in secondary AML/MDS. *Methods.* We selected nine patients with secondary AML/MDS and one Fanconi Anemia patient with MDS with a rearrangement on the short arm of chromosome 6. Karyotypes of G-banded metaphases were described according to ISCN (1995). Metaphase FISH with a panel of 38 DNA clones for 6p12-p25 bands was performed in all cases. Multi-FISH, CGH and FISH with whole chromosome paints and/or centromeric probes were performed in selected cases. *Results.* 6p rearrangements were isolated in 4 patients and included in complex karyotypes in 6. Numerical or structural aberration typically associated with therapy-related AML/MDS, i.e. -5/5q-, -7/7q-, monosomy 18 were respectively found in four, three, and three patients. In three cases full or partial trisomy of the 6p arm was present: i(6)(q10) in one case and dup(6)(p) in two cases. The remaining 7 patients showed 6p unbalanced translocations with diverse chromosome partners or unidentified material. In 3 patients with unbalanced translocations, FISH detected cryptic duplications of a genomic region contiguous to the translocation breakpoints, at band p21, while in two patients a low copy gain with five copies of DNA clones mapping at band p21, were present on der(6) and/or inserted in other derivative chromosomes. In all cases a common over-represented 6p21 region was narrowed to a 5-6 megabase DNA segment extending from the TNF gene to ETV-7. Two patients did not show 6p21 gain. *Conclusion.* 6p21 gains, either as duplication/trisomy or low copy gain, emerged as a new recurrent genomic lesion in secondary AML/MDS with 6p abnormalities. Remarkably, they may be cryptic at conventional cytogenetics and underlie different types of chromosome changes. Putative candidate genes, such as the MHC complex, NOTCH-4, BAK, FANCE, ETV-7, HMGIY and FKBP51, map within the common over-represented 6p21 region. As duplications/low copy gains occurred in both treatment and environmentally induced AML/MDS as well as in the FA patient, toxic insults and congenital instability appear to share the same genetic pathway. *Acknowledgements.* BAC clones were kindly provided by Dr. M Rocchi, University of Bari, Italy.

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0589

CYTOGENETIC AND FISH STUDY IN 203 B-CLL PATIENTS

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Background. The progress in molecular genetic characterization of chronic lymphocytic leukemia (CLL) revealed the prognostic role of IgVH mutational status, of phenotypic changes involving expression of CD38 and ZAP-70, as well as, of chromosomal abnormalities defined by molecular cytogenetic *Methods.* Interphase fluorescence *in situ* hybridization (I-FISH) is able to detect the most common chromosomal abnormalities 13q, 11q, 17p deletions and trisomy 12. *Aims.* The aim of this study was to determine the chromosomal abnormalities in 203 CLL patients using cytogenetic and molecular cytogenetic methods, and to correlate the molecular cytogenetic findings with disease status (stable versus progressive), with immunoglobulin variable heavy chain (IgVH) mutational pattern, and with other clinical parameters. *Methods and patients*. 123 males and 80 females (median of age 62 years) were examined by con-

ventinonal cytogenetic examination on TPA stimulated cells from peripheral blood (167), bone marrow (34) and/or lymph node (2), and by I-FISH on fixed cells. The locus specific and centromeric probes were used (ABBOT-VYSIS) for I-FISH. CGH and M-FISH were used to detect chromosomal changes in patients with complex karyotype. *Results.* Cytogenetic analysis was successful in 127 patients and abnormal karyotype was detected in 35 (28%) patients. Using I-FISH we detected trisomy 12 in 17 (9%) out of 193 analyzed cases, deletion of ATM in 41 (20%) and deletion of p53 in 18 (9%) out of 203 examined cases. Deletion of RB1 was found in 66 (43%) out of 153 analyzed cases and two abnormal clones were revealed in 8 (12%) of them ' one with deletion of only one copy of RB1 gene and the other with deletion of both copies. Two chromosomal abnormalities were detected in 19 patients (9%) – deletion of RB1 together with deletion of ATM gene in 11 of them, and – deletions of RB1 gene together with deletion of p53 in 8 patients in this group. Three abnormalities deletions of ATM, RB1 and trisomy 12 were detected in only one patient. *Summary*. FISH is rapid and sensitive method for determination of chromosomal aberrations of prognostic relevance in CLL patients. The deletion of 13q14 was the most frequent (43%) chromosomal aberration detected by I-FISH in our cohort of CLL patients. The correlation of molecular cytogenetic results with IgVH mutational pattern and with clinical data were analyzed and will be presented.

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0590

A MOLECULAR CYTOGENETIC STUDY OF MANTLE CELL LYMPHOMA AT DIAGNOSIS AND FOLLOW-UP: EVIDENCE FOR A 'TEMPORALLY ORDERED' CYTOGENETIC EVOLUTION?

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Background. Apart from t(11;14)(q13;q32), MCL is also characterized by other nonrandom cytogenetic findings. These additional aberrations are well studied at diagnosis and believed to represent clonal evolution during lymphomagenesis, but little is known about karyotypic changes during the course of the disease. *Methods.* The study included 33 patients with MCL. In all cases, an interphase FISH assay was performed at diagnosis on lymphoma cells from peripheral blood, bone marrow or touch imprints of involved sites. Commercial probes were employed for the detection of t(11;14), +12, 13q-, and abnormalities of ATM, p53, p16, TEL, c-MYC and BCL6 genes. In 14 cases, the same FISH screening was repeated at least once (up to four times) during the course of the disease, at relapse or in the context of partial or no response. *Results.* The most frequent additional findings at diagnosis were ATM deletion in 15 cases (45.5%) and 13q- in 12 cases (36.4%), followed by p16 deletion (3 cases; 1 homozygous), p53 deletion (2 cases), and +12, duplication of the CCND1/IGH fusion gene and BCL6 triplication, in one case each. 11 of the 14 cases studied at follow-up showed karyotypic evolution, with acquisition of p16 deletion (6 cases; 4 homozygous), TEL deletion (5 cases; 2 on the basis of monosomy 12), duplication of the CCND1/IGH fusion (3 cases), p53 deletion (2 cases), and c-MYC amplification (1 case). There was no case with acquisition of ATM deletion, 13q- or +12, but in two cases with 13q- in a minor subclone at diagnosis the aberration was estimated to involve the total of the lymphoma cells at relapse. Interestingly, new BCL6 aberrations were seen in 3 cases (triplication in one and amplification in the other two, including the case with gene triplication at diagnosis) and were detected at the third or the fourth repetition of the screening. The longest survival after detection of these aberrations was 3 months. *Conclusions.* Our data suggest that in most cases of MCL clonal evolution also occurs during the course of the disease, with the acquisition of multiple additional chromosomal lesions. Despite the small number of patients in our series, it seems that some of the aberrations (like ATM deletion or 13q-) are most commonly already present at diagnosis, while others (such as monosomy 12 and/or TEL deletion) appear more often or even exclusively on follow-up. From the clinical point of view, we found that the most informative finding is the overrepresentation of the BCL6 gene, apparently associated with aggressive behavior and perhaps the terminal stage of MCL.

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PROGNOSTIC SIGNIFICANCE OF COMPLEX CHROMOSOMAL REARRANGEMENTS IN PATIENTS WITH CHRONIC MYELOID LEUKEMIA

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Background. Ph chromosome i.e. transolaction t(9;22)(q34;q11) is specific chromosomal aberration in bone marrow cells of patients in chronic phase (CP) of CML. During progression of the disease from the chronic to the accelerated phase (AP) and/or blast crisis (BC), clonal evolution with non-random secondary numerical and structural aberrations is frequently observed. Complex chromosomal rearrangements (CCR) are rather rare and the significance and frequency of different anomalies are poorly understood. *Aims.* The aim of our study was a comprehensive analysis of complex chromosomal rearrangements found in bone marrow cells of 22 patients with CML by molecular cytogenetic methods, determination of chromosomes and chromosomal parts which are involved in CCR during progression of the disease and estimation of frequency of non-random changes if they exist. *Methods.* For the assessment of BCR/ABL fusion gene at the time of diagnosis RT-PCR and/or interphase FISH with locus-specific probe (Abbott-VysisTM) were used (200 interphase nuclei analyzed, cut-off level 2,5% tested on controls). In some patients further molecular analyses were performed by real-time RT-PCR according to EAC protocol using β-2-microglobuline as a control gene. Multicolor FISH (mFISH) was carried out using the '24XCyte' MetaSystems 24 color kit (MetaSystemsTM) to identify precisely complex chromosomal rearrangements in 22 patients. Most of the patients were in the CP at diagnosis. During the course of the disease clonal evolution with complex chromosomal rearrangements appeared in eight patients who remained in CP, two patients progressed to AP and the rest of them to BC. *Results.* The majority of the structural changes were unbalanced. Variant Ph translocations (involving chromosomes 9, 22 and one or more other chromosomes) were found in ten patients, the rest of the cohort had a classical Ph translocation associated with additional structural aberrations. The most frequent chromosomes involved into CCR were found to be Nos. 2 (6x), 7 and 17 (5x), 1, 3, 4 and 5 (4x). Chromosomal regions 1p, 2p, 5q, 7p and 17p were often involved in CCR and the bands repeatedly affected were 17p11.2 (3x) and 7p15 (2x). No one of complex translocation was seen more than once. *Conclusions.* The results of this study demonstrate the very high instability of the genome of malignant cells at the chromosomal level than was expected on the basis of classical cytogenetic analyses. We also proved that CCR are associated with rather poor prognosis and respond poorly to antileukemic treatment. Analysis of CCR by mFISH is important as we believe that such examinations of large cohorts of patients could confirm the significance and non-randomnes of this instability and to find out possible recurrent chromosomal aberrations specific for disease progression. Precise determination of breakpoints on chromosomes involved in CCR of bone marrow cells of CML patients can give new dimension to our understanding of genetic mechanisms which can play role in progression of malignant disease.

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TRANSLOCATION T(9;14)(P13;Q32) IN THREE CASES OF SPLENIC MARGINAL ZONE LYMPHOMA

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Background. Translocation t(9;14)(p13;q32) involving PAX-5 and IgH is an aberration that was first described in lymphoplasmacytic lymphoma (LPL). Nevertheless, new data suggest that t(9;14) is not restricted to a specific morphologic subtype and it is recurrent in other B-cell lymphomas (high-grade and low-grade). Moreover, chromosomal studies in splenic marginal zone lymphoma (SMZL) revealed a high incidence of deletions of 7q, gains of 3q and few incidence of translocations involving 14q32. Reviewing reported cases, only one SMZL patient with a complex translocation t(2;9;14)(p12;p13;q32) was previously referred. *Aims.* The aim of this study was to present the finding of t(9;14)(p13;q32) in SMZL patients (diagnosed by citology, immunophenotype and his-

tology) and to know the implication of PAX-5 gene in this pathology. *Methods.* Among a series of 141 SMZL we applied spectral karyotyping (SKY) in 25 cases with complex karyotype. In patients with t(9;14) we studied by FISH the involvement of PAX-5 gene using a split probe (Dako, Denmark). *Results.* In 3 out of 25 cases with t(9;14)(p13;q32) detected by SKY, rearrangement of PAX-5 was confirmed. Our three patients presented a complex karyotype. The most frequent additional abnormalities were gains of chromosome 1 (3 cases) and gains of chromosome 3 (2 cases). They showed morphology and immunophenotype features typical of SMZL. All three cases presented bone marrow involvement and two showed a splenic diffuse pattern uncommon in this pathology. *Summary/Conclusions*. In all patients t(9;14) was found after the application of spectral karyotyping (SKY) technique confirming that complex rearrangements could mask this anomaly when are studied by conventional cytogenetics. Our findings confirm the rare but recurrent involvement of t(9;14) in SMZL cases and that this anomaly is not specific for a subtype of NHL. The prognosis of PAX-5 rearrangements in SMZL remains unclear and a further follow-up of patients is necessary to better understand the role of this aberration. *Acknowledgements.* This work has been partially supported by grants from Instituto de Salud Carlos III, Ministerio de Sanidad y Consumo (PI 051072) and Fundació La Marató de TV3 (Càncer). We want to thank Juan Cruz Cigudosa for his help in the tone-up of SKY technique and Carme Melero for their expert technical assistance.

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I-FISH ANALYSIS OF IMMUNOFLUORESCENTLY LABELED PLASMA CELLS IN PATIENTS WITH MULTIPLE MYELOMA

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Background. Early detection of specific chromosomal aberrations in plasma cells of patients with MM may have diagnostic, prognostic and therapeutic implication. One of the most frequent and prognostically most significant clonal aberrations in MM are rearrangements of IgH gene at 14q32 region (generally poor prognosis), deletions of RB1 gene at 13q14 and/or loss of whole chromosome 13 (moderately adverse or medium prognosis). The translocation t(11;14)(q13;q32) is associated with longer overall survival and, in contrast to other IgH rearrangements, it is considered to be a favorable prognostic factor. However, the detection of genetic aberrations involved in MM by conventional cytogenetic and/or classical I-FISH methods may be limited by low proliferative index of plasma cells. The sensitivity and specificity of I-FISH analysis may significantly increase previous immunofluorescent labeling of malignant myeloma cells. This method allows identification of chromosomal changes even in cases with low bone marrow infiltration. *Aims.* The aim of the study was to assess the frequency of the most significant chromosomal aberrations (abnormalities of IgH gene and RB-1 gene) in immunofluorescently labeled non-dividing plasma cells of patients with MM by I-FISH and to evaluate their prognostic significance. *Methods.* I-FISH analyses were done on plasma cells labeled by the Amca Anti-Human kappa-chain, Amca Anti-Human lambda-chain and Amca Anti-goat IgG monoclonal antibodies (Vector Laboratories). For I-FISH directly marked locus specific DNA probes (Abbott-Vysis) were used. Detection of deletion/monosomy of chromosome 13 was performed by LSI 13q14 (RB1) and LSI 13q34 DNA probes. Aberrations of 14q32 region were proved by LSI IgH rearrangement probe. For detection of specific IgH translocations LSI IgH/CCND1 and/or LSI IgH/FGFR3 probes were used. Molecular cytogenetic findings were correlated with different clinical and laboratory parameters. *Results.* Altogether 114 newly diagnosed MM patients were examined by I-FISH. Deletion of RB-1 gene was found in 22 (19%) patients and monosomy 13 was identified in other 34 (30%) of them. Combination of both aberrations was proved in 6 (5%) cases. Aberration of IgH gene was found in 60 (57%) from 106 evaluated patients (deletions, partial trisomies and monosomies and numerical changes involving chromosome 14 were also found). Sixteen out of 33 cases (48%) evaluated for t(11;14)(q13;q32) were positive. Another six patients were examined for t(4;14)(p16;q32) and translocation was proved in four of them. Patients with aberration of 13q had significantly shorter event-free survival (EFS), strong association with advanced clinical stages was also proved. Between IgH positive and IgH negative cases, difference in EFS was not statistically significant due to heterogeneity of IgH positive patients. In most cases t(11;14) is associated with other chromosomal aberrations and prognostic relevance of these findings remains to be cleared. *Summary.* I-FISH on plasma cells detected by the immunofluorescent staining permits to increase yield of number of chromosomal abnormalities in MM patients. This method significantly contributes to the higher sensitivity and specifity of diagnostic procedures and is important for determination of prognosis and treatment of MM patients. Our results confirmed 13q aberrations as a marker of poor prognosis (p=0.008).

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DETECTION OF STRUCTURAL ABERRATIONS OF CHROMOSOME 7 IN MYELOID MALIGNANCIES USING COMBINATION OF MOLECULAR CYTOGENETIC TECHNIQUES

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Background. Complete or partial loss of chromosome 7 is a frequent chromosomal aberration in myeloid disorders such as myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML). Allelotypic studies have delineated at least three distinct loci, that are frequently deleted: 7q22, 7q31 and 7q35. It has been hypothesized that there are localized tumor suppressor genes that contribute to the pathogenesis of these disorders. *Aims.* Using combinations of conventional and molecular cytogenetic techniques we have focused on the analyses of deletions and translocations involving chromosome 7 in bone marrow cells of patients with MDS and AML. Correlation of clinical characteristics, outcome and survival of patients according to cytogenetic findings were evaluated. *Methods.* Using classical cytogenetic techniques we examined 32 patients with myeloid malignancies (16 MDS, 16 AML), whose bone marrow cells contained 7q deletion or rearrangements of chromosome 7. Fluorescence *in situ* hybridization (FISH) with locus specific probe for 7/7q31 region (ABBOTT VYSIS), and 7q22/q35 specific probe (QBiogene) were used in all patients to confirm the deletion and to prove the breakpoints. Multicolor banding technique (mBAND) for chromosome 7 was carried out in 16 patients for precise mapping of the extent of deletions (XCyte7 DNA Probe Kit, MetaSystems). Chromosomes involved in complex translocations were identified by multicolor FISH (mFISH) (24XCyte DNA Probe Kit, MetaSystems). *Results.* By using conventional cytogenetic techniques deletion of 7q was found in 5 patients, in two as a sole aberration, in 27 patients translocation of chromosome 7 was ascertained. According to the results of FISH with locus specific probes for 7q22, 7q31 and 7q35 region and mBAND for chromosome 7 five groups of patients were established: patients with deletion 7q as a sole aberration (2x), patients with deletion 7q and complex karyotype (3x), patients with combined translocation and deletion 7q (19x), patients with combined translocation and deletion 7p (5x) and patients with translocation of chromosome 7 without deletion 7p or 7q (3x). Deletions of all three FISH screened regions on the long arms were the most frequent, the breakpoints were heterogenous and varied among patients. On the short arms of chromosome 7 region 7p13.2p15.2 was the common deleted segment. Complex karyotype was confirmed by mFISH in 29 patients. Most of the deletions in patients with complex karyotype were cryptic, not detectable using conventional cytogenetic techniques. *Summary.* Aberrations of chromosome 7 are associated with a poor prognosis, increased risk of infection, rapidly progressive disease and poor response to treatment. Survival time was short in our cohort of patients (median 7 months), 25 patients died. Systematic molecular cytogenetics studies reveal cryptic rearrangements and provide novel information about genes possibly involved in these events.

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T(5;12)(Q23-31;P13) WITH ETV6-ACSL6 GENE FUSION IN POLYCYTHEMIA VERA

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Background. Myeloproliferative disorders (MPD) are chronic clonal proliferations of haematopoietic progenitors. Typical MPDs include chronic myeloid leukaemia (CML), polycythemia vera (PV), essential thrombocythemia (ET) and idiopathic myelofibrosis (IF). Oncogenic alterations identified so far in MPDs target tyrosine kinases, result from chromosomal translocations or gene mutations and lead to constitutive activation of survival and proliferation pathways. Reciprocal translocations lead to gene fusion and production of chimeric proteins such as BCR-ABL, ETV6-PDGFRB or PCM1-JAK2. Point mutations of the JAK2 kinase occur in almost all PV, and in around half of ET and IF. JAK2 functions downstream of membrane receptors, including cytokine receptors such as IL3 receptors. Overproduction of IL3 has been reported in atypical CML following rearrangements of the IL3 gene upstream region in cells from patients with t(5;12)(q23-31;p13) translocation and ETV6-ACSL6 fusion. *Aims*. We report here two cases of t(5;12)(q23-31;p13) translocation with ETV6-ACSL6 rearrangement in PV patients. *Methods and results.* Cytogenetic analysis with R-banding technique on BM cells detected a t(5;12)(q23-31;p13) translocation. We demonstrated the involvement of ETV6 and the 5' region of ACSL6 (previously ACS2) in the translocation by using dual-color fluorescence *in situ* hybridization (FISH) on metaphases of MPD cells from one patient, using labeled-BAC clones. The 5q23 breakpoint was characterised by using two contiguous BAC clones, from centromere to telomere and results showed that the breakpoint was located in the 5' region of ACSL6. We determined the status of the JAK2 kinase in this patient by sequencing DNA from peripheral blood cells. The Val617Phe mutation was not found. *Summery/conclusions*. In conclusion, we have described a genomic alteration that had never been described before in PV patients. As suggested the result of the rearrangement could be an upregulation of the IL3 gene. Due to the orientation of the rearranged genes on the der(12), it is probable that the cause of this upregulation is a chromatin conformation change rather than an ETV6 promoter effect. In one of the patients the sequencing of blood cells DNA failed to show a JAK2 mutation. Two explanations may be proposed. First, because the t(5;12) is only present in 20% of mitoses, the JAK2 mutation could be present in the same clone but not detected by our DNA analysis although its measured sensitivity was below this range; in this case, the translocation would appear as a secondary event. Eosinophilia was detected in one patient with M2 subtype of AML at the second relapse, concomitantly with the occurrence of the t(5;12). Alternatively, increased level of IL3 due to the rearrangement may enhance non-mutated JAK2 activity and trigger PV, eosinophilia and basophilia, and may eventually lead to acute transformation of the translocated clone. If this is the case, genomic events such as a t(5;12) rearrangement may account for few JAK2-negative PV.

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ADDITIONAL CHROMOSOMAL ABNORMALITIES IN PH-POSITIVE CML *DE NOVO*: A MULTICENTER STUDY

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Chronic myeloid leukemia (CML) is a clonal disorder of multipotent haematopoietic cells associated with specific cytogenetic changes involving a translocation t(9;22)(q34:q11) resulting in Ph chromosome occurrence. Cytogenetic investigations revealed that Ph chromosome appeared most often as a sole karyotype aberration during the chronic phase, whereas additional changes frequently accompanied or preceded a transformation to the advanced stages of CML (50-80%). However, the additional cytogenetic changes are found in 10-20% of CML patients at diagnosis and their prognostic impact is still difficult to assess unequivocally. Cytogenetic study as a part of Polish national program of 'Development of standard operating procedures for the diagnosis and follow up of treatment of CML in 2005' was perfomed to evaluate the appearance and the frequency of additional cytogenetic changes in patients with *de novo* CML. *Material and Methods.* A total number of 206 newly diagnosed Ph-positive CML patients investigated in 6 cytogenetic centers formed the subjects of this study. All cases were analyzed by routine cytogenetic techniques on unstimulated and/ or stimulated bone marrow samples according to standard protocols. Karyotypes were described in accordance to the ISCN1995. Additionally, nearly all cases underwent FISH, RT-PCR, and RQ-PCR analysis for BCR/ABL. *Results.* 40 of 206 patients (19,42%) showed aberrations different from simple t(9;22). Constitutional changes [inv(9)(p13q13)] were found only in 2 patients. In the remaining patients, karyotypes showed rearrangements related to leukemia. Three-way Ph translocations were observed in 13 cases (32,5%), involving nonrandom chromosome bands, such as 1q21, 2p13, 3p21, 3q21, 10q23, 10q25, 15p11, and 17q24. Other structural changes, not related to Ph translocation, accounted for 30% of cases (12/40): add(1)(p36), t(1;19)(q32;p13), t(1;17)(p32;p13), add(2)(qter), t(4;22)(p16;q11), t(4;22)(q31;q13),), del(6)(q21), t(7;17)(q32;q25), inv(16)(p11.2q24), del(17)(p13), add(21)(p11), add(18)(p11). Numerical aberrations were also observed in 30% of cases (12/40). Beside the most common trisomy/tetrasomy 8 and +Ph, the aberrations: +X, -Y, +15, +17, +19, -21, +21, were disclosed. Few unidentified markers were also revealed. *Conclusions.* Among described aberrations, we found significant part of nonradom cytogenetic changes described previously, but some of them, to our knowledge, are described for the first time in relation with CML. For all patients with addditional aberrations further observations and at least 1 year follow-up are needed to evaluate their prognostic significance based on clinical stage, prognostic scores and response to treatment.

Genomics and proteomics

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FUNCTIONAL ANALYSIS OF CANDIDATE GENES LOCALISED IN 13Q14.3, A REGION COMMONLY AFFECTED IN B-CLL

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Background. Genomic material from chromosomal band 13q14.3 is lost in a variety of neoplasms. Thus, a tumor suppressor mechanism distinct from the RB1 gene has been postulated in this region. In B-cell Chronic Lymphocytic Leukemia (B-CLL), the most common leukemia in the Western world, deletion within chromosomal band 13q14.3 is the most frequent genomic imbalance. However, the pathomechanism in the critical region has not yet been defined. Characterisation of the function of genes in the critical region will allow identification of the most likely tumor suppressor candidates and their role in tumor pathomechanism. Therefore, we analysed the function of different candidate genes localised in 13q14.3. *Aim.* The aim of this project is functional analysis of candidate genes localised in 13q14 such as RFP2, C13orf1, DLEU2/LEU2/BCMSUN/DLB2 and KPNA3. To this end, expression levels of those genes were modulated by knock down and overexpression followed by subsequent analysis of transcriptome changes. *Methods.* Gene expression was modulated by overexpression using cDNA plasmids and knock down using RNAi. A combined lipofection and electroporation technology was used in order to obtain sufficiently high transfection efficiency in cell lines with loss of 13q14 (Granta 519). RNA was isolated from cells after 4, 7 and 12 hours to check genome wide gene expression levels via oligonucleotide arrays. In a second strategy, we used RNAi technology to knock down RFP2, C13orf1, DLEU2 and KPNA3 in human embryonic kidney cells (HEK-293) and HELA cells. RNA was isolated after 48h to identify effects in downstream target genes via expression profiling. Differentially expressed genes of both strategies were verified using Real-Time-PCR. *Results.* To analyse phenotypic changes in transfected cells, a significant overexpression or knock down of the analysed genes was essential. For RFP2 and C13orf1, we could achieve an overexpression of over 90 fold compared to the transfection of empty vector. Overexpression of DLEU2 was only possible up to 12 fold. In HEK and HELA cells, the knock down of C13orf1 and KPNA3 was over 70%. For RFP2, a 60% knock down could be achieved and for DLEU2, the knock down was between 40 and 50%. Downregulation of KPNA3 by RNAi was also shown on protein level. Using RNAi in mammalian cells specificity of knock down has to be shown. There exists an interferon response mechanism in case double stranded RNA is injected into the cell for example by a virus. To be sure not to induce the interferon response in our siRNA transfected cells, we checked expression level of a marker gene for interferon response (OAS1). Using both strategic approaches, we could identify a number of gene products which are affected by modulation of expression of candidate genes of B-CLL. *Summary.* Candidate genes of B-CLL were modulated in their expression levels and phenotypic effects were analysed by expression profiling genome-wide. Our results suggest involvement of different signalling pathways in the pathomechanism residing in 13q14.

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VALUE OF PROTEOMIC SCREENIG FOR PREDICTION OF GRAFT VERSUS HOST DISEASE AFTER ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Background. We have recently published a polypeptide pattern specific for the early diagnosis of acute GvHD (aGvHD) , based on the application of capillary electrophoresis (CE) and mass spectrometry (MS). *Aims.* Here we report the application of an aGvHD-specific pattern to prospectively and blinded collected samples from 78 patients (33 AML, 10 sAML, 13 ALL, 2 MDS, 6 PCT, 5 SAA, 3CLL, 2 OMF, 2 Hodgkin Lymphoma, 1CML, 1 NHL[mhh]). Fifty patients were transplanted from matched unrelated donors (MUD), 27 received stem cells from matched related donors (MRD), 2 from haplo-identical donors. In the majority of the patients the GvHD prophylaxis was methotrexat (59) or mycophenolate(24) and cyclosporin A, 2 patients received T-cell depleted grafts and 4 received steroids instead of MTX. *Methods.* Urine samples were collected on ice prior to conditioning, weekly until discharge from the ward and monthly thereafter. Immediate freezing of the samples avoids degradation of the proteins/peptides. After thawing and removal of confounding materials, like salts, or molecules larger than 30 kDa, the samples were loaded onto the CE, separated according to their charge and, after ionization, directly analyzed in an electrospray ionization time of flight (ESI-TOF)'MS. This lead to the detection of 500 up to 2500 peptides and proteins in individual samples. *Results.* The polypeptide patterns specific for the detection of acute GvHD grade II or greater were applied to the data from the prospectively and blinded collected samples. A total of 760 samples were evaluated using this set up. In general between 4 and 10 samples were collected and screened after HSCT, whereas the control groups (other diseases) contain only 1 sample per patient. Taken together, 600 samples from patients after HSCT have been prospectively evaluated. The sensitivity was 92.3% with a positive prediction value of development of aGvHD of 83% and the specificity was 94% with a negative prediction value of more then 98%. *Conclusioon.* we have shown that the application of a peptide pattern, consitisting of several differentially excreted peptides allows prediction of the development of aGvHD. Especially patients developing steroid resistant aGvHD show the aGvHD-specific changes very early (about 10 days) priorto clinical symptoms. Taken together a therapeutic startegy using the aGvHD pattern as guidance for an early start of immunosuppression seems justified.

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FINE-MAPPING THE HISTONE CODE AT 13Q14.3, A CRITICAL REGION IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. B-cell chronic lymphocytic leukemia (B-CLL) is the most common leukemia among adults in the Western world. The most recurrent genomic aberration is deletion of a critical region in chromosomal band 13q14.3, which is found in over 50% of cases (Stilgenbauer *et al.* 1998). So far, no tumor suppressor gene has been identified. The minimally deleted region of 13q14.3 harbors protein-coding genes (RFP2, KCNRG and FAM10A4), two long non-coding RNA genes (BCMS and Dleu2/BCMSUN) and two microRNA-genes (mir-15a and mir-16-1). In B-CLL patients, RFP2 is most significantly down-regulated (Mertens *et al.* 2002). Current evidence indicates that the region is regulated by an epigenetic mechanism. In order to identify this epigenetic pathomechanism, we analyzed the histone code, which epigenetically marks genes as being active or inactive, and which could explain their differential expression. *Aim.* In this study we fine-mapped the chromatin of the region 13q14.3 by analyzing DNA- and histone-modifications in order to identify the epigenetic mechanism responsible for the transcriptional deregulation of 13q14.3. *Methods.* We analyzed the chromatin modifications of the region by chromatin immunoprecipitation (ChIP) using antibodies specific for different histone modifications. Specificity was verified by Western blot analysis of precipitated chromatin and the precipitated CpG-islands localized in 13q14.3 were quantified with Q-PCR. *Results.* Chromatin immunoprecipitations showed enrichment of methylated lysine 4 of histone H3 (H3K4) at CpG-islands in the vicinity of the two non-coding RNA genes of 13q14.3. Between the different CpG-islands in the region enrichment of methylated H3K4 differed. Surprisingly, histone modifications also concentrated to specific regions within one CpG-island. *Conclusions.* The CpG-islands localized in the vicinity of the non-coding RNA genes in 13q14.3 show a specific histone code. Interestingly, we find differences between histone modifications and the distribution of DNA-methylation. These differences indicate a regulatory mechanism involving epigenetic modifications that could explain expression patterns of candidate genes in normal and in tumor cells. Our systematic fine-mapping of the histone code in the critical region will show, whether this region is uniformly marked by different histone modifications and to which extent there are differences between the seven CpG-islands in the region.

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0600

XLN PREDISPOSES TO MYELOID MALIGNANCIES BUT GAIN OF FUNCTION WASP MUTATIONS ARE NOT FOUND IN MYELODYSPLASIA OR AML

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Background. X-linked neutropenia (XLN), described in 2001 in a three generation family with 5 affected men, is caused by a T843C WAS mutation, resulting in a L270P gain-of-function mutation GTPase binding domain (GBD) of the Wiskott-Aldrich-syndrome protein (WASP) (Nat genet 2001,27,313). XLN is characterized by recurrent bacterial infections, severe neutropenia and monocytopenia, decreased CD4+/CD8+ ratios, and bone marrow maturation arrest at the promyelocyte/metamyelocyte stage. The mutation disrupts the auto-inhibitory domain of WASP, causing constitutive activation, and leads to neutropenia by an unknown mechanism. Since our original report, only one more case, originally diagnosed as myelodysplasia, has been identified, with an I294T mutation (Blood 2001,98,439a). *Aims.* 1) to extend the description of the XLN phenotype, including 2 cases of myelodysplasia 2) to investigate whether patients with GBD mutations can present with myelodysplasia, AML or monosomy 7. *Methods.* We investigated 206 cases with myelodysplasia (n=49) or acute myeloid leukemia (n=157). There were 173 adults (median age 65y, range 21-89y) and 34 children (<20y, median age 12y, range 2m-20y). 56 Cases had monosomy -7. Male/female ratio was 1.35. There were two brothers with monosomy 7, one with AML M5 and the other with secondary ALL after MDS. Exons 7-10, encoding the GTPase Binding Domain (residues 230-381), were amplified and screened for mutations using dHPLC. DNA from case IV2 [Nat genet, 2001, 27, 313] was used as positive control. *Results.* We observed 2 myeloid malignancies among the 5 affected members in this family. Patient II3 developed a myelodysplastic syndrome (MDS-RAEB) at age 65, while under hematopoietic growth factor support. He achieved stable remission after three courses of decitabine, but died after complicated colon surgery 3 years later. Patient III6 succumbed to refractory MDS RAEB, rapidly evolving to MDS-RAEBt at age 38. In both cases, monosomy 7 was identified in the leukaemic cells. Although immunoglobulin levels are normal in adults, case IV1 had IgA deficiency at age 12mo. At the age of 12 years, he developed auto-immune adrenalitis. Together with the inverted CD4/CD8 ratios that were observed in 3/3 tested cases, this further supports immune dysregulation as part of the phenotype. Reasoning that the maturation arrest at the promyelocyte/metamyelocyte stage can masquerade as myelodysplasia and that XLN can evolve to MDS/AML, we investigated whether GBD mutations occur in myeloid malignancies. In 206 samples from patients with MDS or AML, with or without monosomy 7, no intronic mutations were found in the exons 7-10 of the WAS gene. One mutation was found in intron 6 in a male patient with AML and monosomy 7. The mutation did not influence splicing of the exon and was thus considered irrelevant. *Conclusions.* Immune dysregulation and increased risk of MDS seem to be part of the XLN phenotype. However, GBD mutations could not be demonstrated in this series of paediatric and adult MDS/AML, either or not associated with monosomy 7. Therefore, MDS or AML does not seem to be a presenting feature of GBD mutations of WASP.

0601

INTRONIC MICRO RNA ANALYSIS IN LEUKEMIA REVEALS COORDINATED EXPRESSION WITH HOST GENES

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In leukemia, the integrity of the transcriptome is altered by chromosomal translocations, deletions, duplications, as well as by epigenetic changes in chromatin structure. By targeting mRNAs for translational repression or RNase-dependent hydrolysis (AU-rich miRNAs or shRNA-like effects), the micro RNA (miRNA) component of the transcriptome is estimated to regulate expression of up to 30% of all proteins. Yet the causes and role of deregulated miRNA expression in malignancy are largely unknown, in part because promoter events are not characterized. Since more than one-third of all known mammalian miRNA genes are encoded in the introns of protein-coding genes they may be regulated by the same promoter events that regulate host-gene mRNA expression. To provide experimental validation for coordinated expression of miRNAs and their host genes we compared Affymetrix U133A gene expression data for the promyelocytic NB4 and acute myelogenous leukemia AML2 cell lines with the expression of miRNA precursors. We found similar patterns of host gene expression in the two cell lines and a good correlation with the expression of miRNA precursors in NB4 cells (r=0.464, N=30 miRNAs, $p<0.016$). To further demonstrate that host gene mRNAs and miRNAs are expressed from common transcripts, we activated promoter events by enforcing the expression of Lyl1 a basic helix-loop-helix transcription factor that is often over-expressed in AML. This resulted in a greater than 2-fold increase in hsa-mir-126-1, 032-2, 107-1, 026a, -023b, -103-2, and 009-3-1 intronic miRNA precursors and a corresponding increase in host gene expression. Meta-analysis of microarray data across many experiments and platforms (available through Oncomine.org) has been used to study the cancer transcriptome. To help determine if intronic miRNAs play a substantial role in malignancy, we correlated host gene expression data with the expression of predicted miRNA targets. Less than 20% of all differentially expressed genes in leukemia and lymphoma were predicted targets, compared to 68% in breast cancer. Since the Gene Ontology term *ion binding* is most commonly associated with miRNA host genes, the data suggest that this cancer module is relatively inactive in leukemia and lymphoma, compared to breast cancer. Gene cluster analysis of a leukemia data set using only miRNA host gene expression was able to classify patients into similar (but not identical) subsets as did an analysis based on over 20,000 transcripts. To further demonstrate that miRNAs and their host genes are expressed from the same transcription unit, we correlated the expression of miRNA targets with that of genes that are either hosts for miRNAs or are situated several kilobases downstream of a miRNA, and thus belong to different transcription units. We applied this analysis to a subset of 81 AML patients that presented a normal karyotype and found significant negative correlations ($p<0.01$) between the levels of host genes for hsa-mir-15b, -103-1, and -128 and the expression ranks of their predicted gene targets, but no statistically significant correlation between non-host genes and targets for up-stream miRNAs. These data demonstrate co-regulated expression of host genes and intronic miRNAs and the usefulness of intronic miRNAs in cancer profiling.

0602

IN VITRO AND *IN VIVO* VALPROIC ACID RESPONSE SIGNATURES IN ADULT ACUTE MYELOID LEUKEMIA

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Background. Recently, inhibitors of histone deacetylases (HDACIs) like valproic acid (VPA) have been shown to display activity in murine leukemia models, and to induce tumor-selective cytoxicity against blasts from patients with acute myeloid leukemia (AML). While there exists some knowledge regarding the potential function of HDACIs, there remain many unsolved questions like e.g. what factors determine whether a cancer cell undergoes cell cycle arrest, differentiation, or death in response to HDACIs. In addition, most studies evaluated HDACIs as single agent *in vitro*. Thus, there is still limited data on HDACIs effects *in vivo*, as well as HDACIs function in combination with standard induction chemotherapy. *Aims.* Following determination of VPA response signatures in different myeloid leukemia cell lines and in primary AML blasts *in vitro*, we next sought to analyze *in vivo* VPA effects in blasts from adult *de novo* AML patients, entered within two randomized multicenter treatment trials of the German-Austrian AML Study Group. *Methods.* Using DNA Microarray technology, we profiled gene expression in order to determine VPA *in vitro response signatures* following 48 hours VPA treatment of myeloid leukemia cell lines (HL-60, NB-4, HEL-1, CMK and K-562) and primary AML blasts. Next, in an initial attempt we evaluated the VPA effects on gene expression in AML samples (n=18) collected within the AMLSG 07-04 trial for younger (age<60yrs) and within the AMLSG 06-04 trial for older adults (age>60yrs), in which patients are randomized to receive standard induction chemotherapy (idarubicine, cytarabine, and etoposide = ICE) with or without concomitant VPA. Gene expression was profiled in AML blasts collected at the time of diagnosis and following 48 hours of treatment with ICE or ICE/VPA. *Results.* In accordance with previous studies *in vitro* VPA treatment of myeloid cell lines induced the expression of the cyclin-dependent kinase inhibitors CDKN1A and CDKN2D coding for p21 and p19, respectively. In general, supervised analyses using SAM (Significance analysis of Microarrays) revealed many genes known to be associated with a G1 arrest. In all cell lines except for CMK we examined an up-regulation of TNFSF10 coding for TRAIL, as well as differential regulation of other genes involved in apoptosis. Furthermore, gene set enrich-

ment analyses showed a significant down-regulation of genes involved in DNA metabolism and DNA repair. First results from our ongoing analysis of *in vivo* VPA treated samples are encouraging as we e.g. also see an induction of CDKN1A expression. However, the picture observed is less homogenous as concomitant administration of ICE, as well as other factors like e.g. VPA serum levels might substantially influence the *in vivo* VPA response. *Conclusions/Perspectives.* While our findings support previous findings, our data are likely to provide new insights into the VPA effect *in vivo*, and this study may proof to be useful to predict AML patients, which might benefit from VPA treatment. To achieve this goal, we are currently analyzing more samples, and we are aiming to correlate gene expression findings with histone acetylation status, VPA serum levels, cytogenetics, molecular genetics, and clinical data.

0603

GENOMIC APPROACH TO UNDERSTAND THE MECHANISMS INVOLVED IN BCR-ABL-MEDIATED RESISTANCE TO APOPTOSIS

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Background. Bcr-Abl is an oncoprotein involved in malignant transformation and resistance to apoptosis, processes that are not fully understood. *Aims.* To investigate the impact of enforced expression of Bcr-Abl on global gene expression and apoptotic machinery. *Methods.* Ectopic expression of Bcr-Abl was obtained using retroviral vectors. Apoptosis was evaluated against different stimuli by flow cytometry (annexinV/PI and DNA content). Expression of apoptotic genes was examined by RT-PCR and Western-blot. Global gene expression of wild-type and Bcr-Abl+ cells was performed with oligonucleotide microarrays. Differentially expressed genes were identified based on log-ratios consistently above/below intensity-dependent cutoff curves obtained by applying HTself method to self-self data. *Results.* Microarray analysis of Bcr-Abl transfectants identified 465 common genes that were overexpressed and 70 underexpressed in cells that became resistant to apoptosis. The upregulated genes are mainly related to cell motility, communication, growth, death, signal transduction and metabolism. Among them, genes involved in apoptosis and immune system regulation such as faim, caspase-9, nfat, and prame were found. Most of the underexpressed genes are related to signal transduction pathways (tyrobp, calcineurin A β, and traf-3). Bcr-Abl expression conferred resistance to death-inducers in HL-60 and HeLa cells, but not in SKW6.4 cells. Protein levels of Bcl-xL, Mcl-1 and Flip were higher in HL-60/Bcr-Abl than in HL-60. These effects were not observed in HeLa.Bcr-Abl and SKW6.4.Bcr-Abl. In contrast, pro-apoptotic protein Bid was reduced in HL-60/Bcr-Abl and SKW6.4/Bcr-Abl. *Conclusions.* In sum, ectopic expression of Bcr-Abl is capable of protecting HL-60 and HeLa, but not SKW6.4 cells from apoptosis, suggesting that the cellular context is also important to Bcr-Abl cell malignant transformation and apoptosis resistance. The differentially expressed genes identified deserve further investigation in order to evaluate their potential as therapeutic targets or prognostic markers for CML. Supported by: FAPESP, CNPq, Institute for Investigation in Immunology-Millennium Institute/CNPq and SBIBAE.

0604

NOVEL TRANSMEMBRANE MEGAKARYOCYTE PROTEINS IDENTIFIED BY COMPARATIVE GENE EXPRESSION PROFILING OF *IN VITRO* DIFFERENTIATED HUMAN MEGAKARYOCYTES AND ERYTHROBLASTS

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We have used cDNA microarrays to investigate the transcriptomes of megakaryocytes (MKs) and erythroblasts (EBs) to identify genes that are differentially expressed between the two cell types. MKs and EBs have a common, bipotent progenitor and we hypothesised that the subset of genes that are differentially expressed between them might contain novel 'lineage specific' elements. CD34+ Haematopoietic Stem Cells (HSCs) isolated from Umbilical Cord Blood were purified by magnetic cell separation. For differentiation into MKs, HSCs were cultured for 7 days in serum-free medium containing Thrombopoietin (TPO) and IL-1β. For differentiation into EBs, HSCs were cultured for 10 days with Erythropoietin (EPO), IL-3 and Stem Cell Factor (SCF). Differentiated cells were purified by Fluorescence Activated Cell Sorting (FACS) to greater than 95% purity prior to RNA isolation. RNA was amplified and labelled using the SMART Template-Switching PCR protocol and the gene expression profiles of MKs and EBs were then directly compared using cDNA microarrays containing 15,000 features (Sanger Hver2.1.1.). Twenty hybridisations were performed, representing five biologically paired comparisons, each with four technical replicates. Statistical analysis of this data identified 635 features that were upregulated in MKs (>2-fold, $p<0.05$), and 219 features in EBs. Known lineage specific markers, such as ITGA2B (CD41), GP9 (CD42a) and GP1B_ (CD42b), were upregulated in MKs relative to EBs as expected. In addition, a number of novel transmembrane proteins were also identified as upregulated in MKs. RT-PCR was used to validate the differential expression of both known and novel transcripts and to investigate the expression of these genes in other haematopoietic lineages. The presence of selected transmembrane proteins on platelets and MKs was confirmed using murine anti-sera generated against recombinant, E. coli expressed protein. Gene silencing experiments in Zebrafish and human MKs are in progress to determine the biological role of these novel proteins.

0605

IDENTIFICATION OF METHYLATED PROMOTERS IN THE OCI/AML2 ACUTE MYELOGENOUS LEUKEMIA CELL LINE USING CPG MICROARRAYS

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Background. Aberrant gene expression is characteristic of acute myelogenous leukemia (AML). The mechanisms responsible for this altered state of transcription are cytogenetic alterations such as chromosomal deletions, translocations and amplifications that have been broadly studied in the context of AML, post-transcriptional gene silencing (siRNA/miRNA) and epigenetic alterations that include modifications in the histone code and altered patterns of DNA methylation that lead to transcriptional gene silencing in particular of tumor suppressors. In recent years several efforts have been made to identify genes that are silenced through DNA methylation mostly on the basis of candidate tumor suppressor detected with other techniques. Here we report the results of a genome-wide screen of methylated CpG islands and its correlation with transcriptionally inactive loci in the genome of the OCI/AML2 cell line using microarray technology. *Aims.* The aim of this study is to identify, in an unbiased way, hypermethylated and trascriptionaly inactive sites in the genome of the human OCI/AML2 cell line using microarray technology and a comprehensive bioinformatics approach. *Methods.* We used an anti-5-methyl-cytosine antibody to pull down methylated sequences from sonicated genomic DNA from the OCI/AML2 cell line. The immunoprecipitated DNA was labeled and hybridized onto the University Health Network (UHN) 12K CpG array (www.microarray.ca) and after visual inspection positive clones were identified. These clones were then intersected with predicted promoters, CpG islands and fist exons (First EF) using the UCSC table browser intersection function. After this filtering step the clones were mapped to the chromosome ideogram using KaryoView (www.ensembl.org). Expression profiling of the same cell line was done using the Affymetrix U133A array. Under-expressed genes were also mapped to the chromosome ideogram using KaryoView. Statistical analysis of overrepresented cytogenetic bands was done using DAVID 2.1. *Results.* After careful analysis and filtering of uninformative clones we found a good correlation between hypermethylated regions of the genome and regions with relative transcriptional inactivity. Some previously reported genomic loci were also found using this technique; such as the 11q22-23 tumor suppressive locus that has been found to be methylated in nasopharyngeal carcinoma and deleted in CLL. Other sites identified using this screen include the 19q13 locus, that has been reported to be methylated in acute lymphoblastic leukemia and a common site for loss of heterozygocity in prostate cancer and the 6p21 locus that has been found to be hypermethylated in normal ageing. *Conclusion.* Global methylation analysis using CpG microarray technology coupled with gene expression data together with bioinformatics has the potential to detect tumor suppressor regions in the genome of cancer cells. The use of this technique will further the understanding of the epigenetic processes involved in abnormal gene regulation in AML. This technique may also prove to be useful in the classification of the disease.

0606

EXPRESSION SIGNATURE OF GENES ASSOCIATED WITH TELOMERE-TELOMERASE COMPLEX IN PATIENTS WITH MYELODYSPLASTIC SYNDROMES AND ACUTE LEUKEMIA: TEP1 GENE IS SURPRISINGLY UPREGULATED IN PROGRESSION OF MDS AND IN LEUKEMIC CELLS

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Background. Knowledge of dynamics of telomere-telomerase complex brings important sign into molecular background of leukemogenesis. Misbalance initiated by erosion of telomeres may affect also expression level of genes enrolled in regulation of telomere length and telomerase activity. Thus, data on expression profiles of associated genes: hTERT encoding catalytic sub-unit of telomerase, the tankyrase (TNKS), TRF1 (Telomeric Repeat binding Factor 1), POT1 (Protection Of Telomeres 1), TEP1 encoding telomere associated protein, and myc may be valuable from the viewpoint of disease prognosis and monitoring of therapy effectiveness. *Aims.* To ascertain expression variations of genes involved in regulation of telomere-telomerase complex in patients with myelodysplastic syndromes (MDS), acute myelogenous leukemia (AML) from MDS, and primary untreated AML with the aim to evaluate their significance as prognostic factors of MDS evolution towards overt leukemia and markers of leukemic cells. *Methods.* The study was done on mononuclear bone marrow (BM) or peripheral blood (PB) samples from 42 patients with MDS, AML from MDS and untreated primary AML divided into subgroups according to the FAB criteria. Mononuclear cells of 14 healthy BM or PB progenitor cells healthy donors served as normal controls. RNA was extracted using modified method of Chomczynski. Relative expressions of hTERT, TNKS, TRF1, POT1, TEP1, and myc RNA were assayed by real-time RTPCR with specific Taq-Man probes in RotorGene 3000A (Corbett Research) in comparison to expression of the housekeeping gene. Results with the ratio higher than mean + 2 s .d. of healthy controls were postulated as cases with positive gene expression. Expression signatures were discussed together with telomere length, telomerase activity and clinical features: proportion of blast cells, results of the DFS analysis and also with individual patients risk score established for MDS according to the International Prognostic Scoring System (IPSS). *Results.* Notable increase of expression of hTERT, TEP1, and POT1 genes was observed in patients with advanced form of MDS (RAEB and RAEB-t) in contrast to insignificant changes of telomerase activity representing a later event in misbalance of telomere-telomerase complex. Significant correlation between individual values of POT1 gene expression and telomerase activity confirmed in MDS and AML patients (p=0.0079) supports role of the POT1 gene as positive molecular regulator of telomerase. On the other hand, no relationships were found between POT1 expression and the IPSS risk score of MDS patients on one side and portion of blast cells in BM/PB both in MDS and AML on the other side. *Summary/Conclusion.* We showed that hTERT and POT1 genes up-regulated already in early forms of MDS and its expression has increasing trend with disease progression. Significantly increased expression of these genes is also feature of mononuclear BM/PB cells of majority of patients at diagnosis of primary AML. These observations predestine POT1 and hTERT genes at least as additional prognostic factors of MDS and molecular markers of AML. High TEP1 expression in patients with advanced forms of MDS and AML indicates on its more active role in signaling of telomere-telomerase complex as it has been supposed.

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0607

TEN NOVEL MUTATIONS IN THE HMBS GENE RESPONSIBLE FOR ACUTE INTERMITTENT PORPHYRIA

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Background. Acute intermittent porphyria (AIP) is an autosomal dominant disorder caused by a partial deficiency of hydroxymethylbilane synthase (HMBS), the third enzyme in the heme biosynthetic pathway. Clinical features of the disease are intermittent attacks of neurological dysfunction, including abdominal pain and neuropsychiatric symptoms. Most of the affected individuals remain asymptomatic throughout their life but 10-20% presenting severe acute attacks. Diagnosis of AIP is often difficult if based on urinary overproduction of porphyrin precursors ALA and PBG only. The erythrocyte HMBS activity is not extensively available and not always informative because of the overlap between the normal and carriers range. The molecular analysis of HMBS gene represents the most reliable diagnostic tool for AIP. The human HMBS gene maps to chromosome 11q24.1-q24.2 with a total of 15 exons. Two distinct promoters direct housekeeping and erythroid specific mRNAs by alternative splicing. So far, more than 210 different mutations have been identified world-wide in the HMBS gene as responsible for AIP, showing a high genetic heterogeneity. Most of the reported mutations have been detected only in single families, however a prevalence of specific mutations in different geographic areas has been reported. Only preliminary data are available for the Italian population. *Aims and Patients*. In this study we searched for molecular defects in HMBS gene, in order to identify the most common HMBS mutations in Italian subjects affected by AIP. We investigated twelve unrelated patients and their relatives. The diagnosis was based on clinical manifestations, elevated urinary excretion and reduced erythrocyte HMBS activity. *Methods.* The promoters, the entire coding region and the intron-exon boundaries of HMBS gene has been amplified by polymerase chain reaction and submitted to direct automated sequencing. Restriction fragment length polymorphisms, poly-acrylamide gel electrophoresis and XL PCR were performed to confirm the presence of putative mutations. *Results.* Twelve different molecular defects in HMBS gene have been identified. Two missense mutations (77 G>A and 962G>A) previously reported and ten mutations are new findings: five deletion, one insertion, one splicing defect, one nonsense and two missense. The 447-467del21bp causes the loss in frame of seven aminoacids in the exon 9 and the13890bp deletion causes the loss of the entire HMBS gene. The 181delG, the 418-419delAA, the 468-470delAA and 652insG mutations cause frameshift and protein truncation at aminoacids 96, 208, 207 and 249 respectively. The loss of exon 6 is due to the IVS5-1G>A splicing defect; the nonsense mutation (940C>T) in exon 15 is responsible for creation of a stop codon at aminoacid 314; two missense mutation (242T>C and 1075 G>A) in exon 6 and 15 result in a Leu 81Pro and Asp359Asn amino acid substitution respectively. *Summary.* These results allowed the identification of ten novel HMBS mutations. In a previous work, we have identified other 11 new molecular defects for a total of 21 new different mutations restricted to the Italian population. This study confirmed the high heterogeneity of molecular abnormalities responsible for AIP phenotype and the presence of clusters of mutations in particular geographic areas.

0608

ASSOCIATION OF HUMAN PLATELET ALLOANTIGENS 1, HPA2, HPA,3 HPA4, AND HPA5 ALLELES AND GENOTYPES WITH SICKLE CELL ANEMIA

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Background. Insofar as sickle cell anaemia (SCA) was described as a hypercoagulable state where occlusive vascular complications (OVC) and progression to stroke are frequently seen, polymorphisms of human platelet alloantigen (HPA) were reported as risk factors for several vascular anomalies, including stroke. With the exception of a lone report documenting association of HPA-5b with SCA OVC, studies on potential association of HPA1 through HPA5 with SCA are lacking. *Aims.* This study investigated the prevalence of HPA1, HPA2, HPA3, HPA4, and HPA5 alleles and genotypes among Bahraini SCA patients and control subjects. Linkage disequilibrium analysis will be used to investigate the disease association of the these polymorphisms. Method. This was a case control study. Study subjects comprised 135 SCA patients (mean age 15.8±9.8) and 187 healthy controls (mean age 27.8±15.1); all were Bahraini nationals. Mutation analysis was assessed by PCR-SSP analysis. Statistical analysis was performed on SPSS v. 13.0 statistics software, significance being set at $p < 0.05$. *Results.* The distribution of HPA2 (p=0.225) and HPA4 (p=0.075) genotypes were comparable between SCA patients and controls. In contrast, higher frequencies of HPA1a (Pc < 0.001), HPA3a (p=0.007) were found among controls, while HPA3b (p=0.034) and HPA5a (P < 0.001) alleles were more frequent in patients. Whereas HPA 3a/3a (p=0.036; RR = 0.463) and HPA 5b/5b (p<0.001; RR = 0.182) were more prevalent among controls, HPA 1b/1b (p<0.001; RR = 19.958), HPA 3b/3b (p=0.042; RR = 1.734), and HPA 5a/5b (p < 0.001; RR = 3.073) were significantly higher among SCA patients. Significant linkage disequilibrium were noted between HPA alleles, with the strongest occurring between HPA1b and HPA5a (_ = 0.119; p< 0.001). *Summary/Conclusion.* Differential association of HPA polymorphism with

SCA was noted among Bahraini patients, with HPA1, HPA3, and HPA5 representing genetic risk factors of SCA. In view of the reported link between HPA polymorphism and OVC, our results serve a diagnostic/prognostic role in identifying SCA patients with possible OVC complications, as well in the development of future therapeutic regimen.

0609

GENOTYPE AND CLONAL EVOLUTION IN CHILDHOOD ACUTE LEUKEMIA CASES

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Background. Leukemia is the phenotypic result of multiple events, which can accumulate in a pre-leukemic clone and whose origin can be either pre-natal or post-natal in different leukemia subtypes. The understanding of consequential events is important for the comprehension of the pathogenesis of pediatric acute leukemia. The aim of our study was to search for hidden genetic aberrations by detecting genome-wide loss of heterozygosity (LOH) and genes copy number variation (CNV) with single nucleotide polymorphism (SNP) arrays. Here we report the dissection of genotype and clonal evolution in two cases of childhood leukemia, a twin pair affected by TEL-AML1 positive ALL and a FLT3-ITD positive AML patient, as a paradigm for describing different mechanisms of clonal evolution. *Results.* the TEL-AML1 positive monozygotic twins with concordant ALL were classified as pre-B ALL and common-ALL. They shared one common Ig/TcR rearrangement amongst others. Both the diagnosis and remission samples analyzed by GeneChip Mapping 10K SNP array showed LOH at the 2q13-14.3 region in both twins, while deletion of the normal TEL allele was only found in twin 2. LOH was not associated with CNV, implying a recombination event resulting in uniparental isodisomy (UPD). Further analyses are necessary to understand the functional implications of this chromosomal abnormality. One hypothesis could be that the twins were born with a genetic predisposition to develop leukemia, along with the t(12;21) and additional events were then responsible for the overt leukemia. UPD of this region has not been reported in other tumors or in remission samples of leukemia. Moreover, this is the first report on constitutional UPD in leukemia patients. We also studied clonal evolution in a FLT3-ITD positive childhood AML patient, who experienced two relapses and for whom we had the availability of the cord blood (CB) sample. The patient was diagnosed at 6 years of age with AML-M1, a normal karyotype and FLT3-ITD mutation. The same FLT3-ITD clone re-emerged at relapse, three months after auto-BMT. The DNA from CB was negative for the FLT3-ITD RQ-PCR, consistent with the well established hypothesis that FLT3-ITD mutations represent post-natal events. The GeneChip Mapping 10K SNP array analysis on DNA from the first relapse showed deletion of the long arm of chromosome 9, and LOH on the whole chromosome 13 not associated with CNV. This latter is consistent with UPD, so either non-disjunction or somatic recombination has led to the homozygosity of FLT3-ITD at 13q14. This is emerging as a frequent event of disease progression, subsequent to FLT3-ITD heterozygous mutation. 10K SNP array analysis did not reveal LOH or copy number changes in the diagnostic and in CB samples. Other methods must be applied to find the primary event(s) giving rise to leukemia in association with FLT3-ITD mutation. *Conclusions.* despite the heterogeneity of the cases presented here, this study shows that genome-wide LOH analysis by SNP arrays represents a powerful tool to unravel cryptic genetic aberrations and to better understand the genetic events cooperating in clonal evolution.

0610

IDENTIFICATION OF NOVEL THERAPEUTIC TARGETS IN ACUTE MYELOID LEUKAEMIA USING A PHOSPHOPROTEOMIC STRATEGY

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Background. Pharmacological inhibition of the dysregulated Bcr-abl tyrosine kinase has emerged as an effective therapeutic strategy in chronic myeloid leukaemia. Although accumulating evidence demonstrates the importance of the constitutive activation of signalling pathways in acute myeloid leukaemia (AML) the development of targeted therapies has been compromised by our limited understanding of the identity of the dysregulated tyrosine kinases in AML. *Aims.* Reasoning that since protein tyrosine phosphorylation is an important mechanism mediating the transduction of proliferative and survival signals we have utilised a phosphoproteomic strategy to identify dysregulated phosphoproteins in myeloblasts from patients with AML.*Methods.* Using an anti-phosphotyrosine antibody we have immunoprecipitated proteins from AML blasts, separated proteins by SDS PAGE and identified proteins within distinct bands by mass spectrometry. Results in primary AML blasts have been compared with CD34+ progenitors from GCSF mobilised normal donors. The methodology was validated using vanadate-stimulated HL60 cells. *Results.* 10 patients with a median age of 51 (range 16-90) were studied. 6 patients had a normal karyotype, one good risk cytogenetics and three adverse risk cytogenetics. Mutations in the flt-3 tyrosine kinase gene were present in four patients. Myeloblasts from every patient demonstrated phosphorylation of MAP kinase (MAPK) implying activation of the ras-MAPK cascade. In contrast CD34+ cells isolated from normal donors demonstrated weak or no MAPK phosphorylation. Since each patient demonstrated MAPK phosphorylation irrespective of flt-3 status we next examined their phosphotyrosine-proteome. This identified a number of phosphorylated and non-phosphorylated proteins in signalling complexes in anti-phosphotyrosine immunoprecipitates present in AML blasts but not normal CD34+ progenitors. These included receptors (ephrin type A-3, interleukin-13), signalling intermediates (TAPP1, RASA1, LARG, cortactin, CD-2 associated protein) and transcription factors (ELK-1, HFK-1). The phosphorylation of a number of the identified proteins was confirmed immunochemically. These proteins identify three functionally distinct groups in AML blasts that were not detected in CD34+ progenitors:(i)intermediates in PtdIns-3-kinase-mediated signalling presumably suppressing apoptosis (ii) intermediates of a tyrosine kinase- mediated ras-MAPK signalling proliferative cascade and (iii) regulators of the actin cytoskeleton and thus cell movement. This strategy also identified a novel pattern of phosphorylation of the 5-HT3A receptor in AML blasts raising the possibility that this protein plays a role in the pathogenesis of AML. Summary: Adoption of a phosphoproteomic methodology has identified novel phosphoproteins in AML which require further validation. Intriguingly our data also point to a common intracellular signalling pathway (ras-MAPK) in AML. These observations provide information for the rational development of targeted therapies in AML.

0611

EXPRESSION OF TGFB1 GENES AND THEIR RECEPTOR TYPES I, II AND III IN LYMPH NODES FROM PATIENTS WITH NON-HODGKINS LYMPHOMAS

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Background. Transforming growth factor β /TGFb/ is a multifunctional cytokine triggering different physiological situations: cell-cycle control, hematopeosis control, cell differentiation, angiogenesis, immunological functions control, apoptosis induction as well as extracellular matrix formation. The TGFb activity depends on the presence of three specific cell surface receptors involved in a variety of important cellular functions. A consequence of TGFb prevalence in an organism is its significant influence on the majority of physiological and pathological processes, including the development of malignant diseases where the lack of TGFb'dependent growth control may be responsible for oncogenesis. Recently there has been a growing number of reports on increased expression of TGFb genes in certain tumors. TGFb1 is most commonly present in humans. *Aims.* The aim of our study was to compare the expression of TGFb1 and its I, II and III receptors in the lymph nodes of 47 never-treated Non-Hodgkin's lymphoma patients in different stages of the disease (15 of these patients were diagnosed with the aggressive lymphoma, 8 with the mantle cell lymphoma and the remaining 24 with the indolent lymphoma; the control group consisted of 7 patients with persistent chronic lymph node enlargement). *Methods.* The QRT-PCR method was employed to assess the activity of TGFb1 and its receptor types I, II and III. *Results.* The expression of TGFb_ differed between the mantle cell lymphoma and the indolent lymphoma groups (p=0,05019). No difference in the expression of TGFb1 was found between any other groups. Also no difference in the expression of genes for TbRI and TbRIII was found in any of the groups, including the control group. A difference in the expression of genes for TbRII was found between the mantle cell lymphoma and the indolent lymphoma groups (p=0,050187). Strong positive correlations were found within the studied subgroups for all the examined parameters for the mantle cell lymphoma and the indolent lymphoma subgroups. In the aggressive lymphoma group we found no positive correlation only for TGFb1 and TbRI as well as TbRI and TbRII. In the control group we found no correlation for TGFb1 and TbRI as well as TGFb1 and TbRIII. For all the remaining parameters strong positive correlations were found. *Conclusions.* Where expressed, TbRIII is the most abundant TGFb receptor and classically functions by binding the TGFb ligand and transferring it into its signaling receptors, TbRI and TbRII. TbRI initiates intracellular signaling by phosphorylating a family of transcription factors, the Smads. In this respect, the lack of positive correlation between TGFb1 and TbRI as well as TGFb1 and TbRIII and the simultaneous strong positive correlation between for TbRIII in all the studied lymphoma subgroups found in our study seem very interesting. It signals an important role the expression of receptor III may play in the pathogenesis of Non-Hodgkin's lymphoma.

Non-Hodgkin's Lymphoma - Experimental

0612

GENE EXPRESSION PROFILING OF PRIMARY HODGKIN/REED-STERNBERG CELLS AND THEIR RELATIONSHIP WITH PRIMARY MEDIASTINAL B-CELL LYMPHOMA

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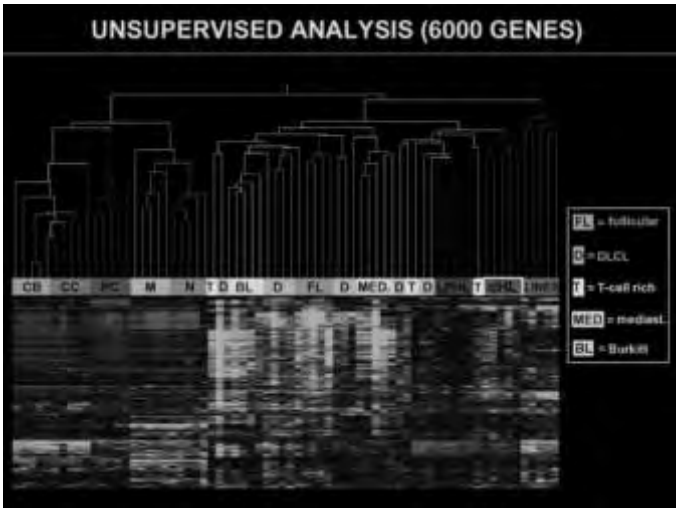
Background. Classical Hodgkin lymphoma (cHL) and PMBCL share some similarities in terms of clinical presentation, histological and immunophenotypical picture, and genetic and pathogenetic features. So far, genome-wide expression profiling studies have compared whole biopsies of PMBCL and other diffuse large B-cell lymphomas (DLCLs) to cHL cell lines, owing to the rarity of primary HRS cells in lymph nodes involved by cHL. These studies reported for PMBCL a partial molecular overlap with cHL cell lines, which was more pronounced than that with other DLCLs. However, cultured HRS cells most likely do not reflect primary HRS cells in all their important features, as they were derived from patients with end-stage disease and from sites (e.g. pleural effusions, peripheral blood) which are very rarely involved by cHL and in which the dependence on the prominent inflammatory background typically surrounding primary HRS cells in the lymph node is lost. *Aims.* to investigate the genome-wide expression profile of primary HRS cells and its relationship with that of other lymphomas (including PMBCL) and of normal peripheral B-cell subsets. *Methods.* ~1000-2000 HRS cells are laser-microdissected from H&E-stained frozen sections of cHL samples. After two rounds of *in vitro* linear amplification, RNA is hybridized to Affymetrix HG-U133 Plus 2.0 chips (interrogating ~54000 probe sets corresponding to ~30.000 genes). Expression profiles are also generated from similar cell numbers of: i) neoplastic cells FACS-sorted from HL cell lines or microdissected from various non-Hodgkin lymphomas, including PMBCL, and from lymphocyte-predominant HL (LPHL) cases; and ii) normal mature B-cell subsets (plasma cells and naïve, memory and germinal center B cells) that are MACS/FACS-sorted from tonsil or peripheral blood of healthy donors. *Results.* Unsupervised hierarchical clustering (see Figure) of the 71 samples so far investigated groups the 25 normal B-cell samples separately from the 46 tumor samples (41 biopsies and 5 HL cell lines), suggesting that the different isolation methods (microdissection vs sorting) do not significantly affect the clustering pattern.



The further branching of the dendrogram shows that each of the four B-cell subsets tend to form discrete clusters, and that, among tumor samples, cell lines grouped apart from primary cases. The latter further split in two sub-branches: one with PMBCLs, Burkitt lymphomas, follicular lymphomas (each of the three forming its own sub-cluster) and DLCLs, and the other branch mainly comprising HLs (with both cHLs and LPHLs tending to form discrete sub-clusters) and T-cell rich B-cell lymphomas. A supervised comparison of primary HRS with HL cell lines shows a highly differential expression (≥ 4fold change) of ~1200 genes, includ-

ing many involved in intercellular signaling, chemotaxis, and immune/inflammatory response. *Conclusions.* These preliminary results suggest that expression profiles can be reliably generated from small numbers of microdissected cells, and that primary HRS cells and HL cell lines seem to differ in various biological features. A more complete analysis (e.g. relatedness of HL with other lymphomas, including PMBCL, and with normal B-cell subsets) will be presented at the Meeting.

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0613

PERIFOSINE, AN ORAL BIOACTIVE NOVEL AKT INHIBITOR, INDUCES ANTITUMOR ACTIVITY IN WALDENSTROM MACROGLOBULINEMIA

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Background. Waldenström's Macroglobulinemia (WM) is an incurable low-grade lymphoplasmacytic lymphoma. The PI3K/AKT pathway is a critical regulator of cell survival by stimulating cell proliferation and inhibiting apoptosis. Our previous studies using proteomic analysis have demonstrated upregulation of members of the PI3K/AKT pathway in WM. The new AKT inhibitor, perifosine (NSC 639966; Keryx Biopharmaceuticals, NY) has demonstrated activity in other B-cell malignancies. *Aims.* We hypothesized that the Akt inhibitor, perifosine will induce cytotoxicity in WM. *Methods.* WM cell lines (BCWM1 and WSU-WM) and IgM secreting low-grade lymphoma cell lines (MEK1, RL) were used. Primary CD19+ malignant cells were obtained from patients after informed consent. Inhibition of proliferation was measured using the MTT proliferation assay. DNA synthesis was measured using the thymidine uptake assay. Apoptosis was determined using Apo2.7 flow cytometry analysis (Beckman Coulter Inc., CA). Bone marrow (BM) stromal cells confer growth and resistance to conventional treatments. We therefore, tested the effect of perifosine on WM cells co-cultured with BM stromal cells. Cell cycle analysis was performed using flow cytometry with PI staining (Molecular Probes, Oregon). IgM secretion was tested using ELISA assay (Immuno-tek, NY). Immunoblotting for pAKt, pERK1/2 and pJNK was performed at 6 hrs of treatment. A two-sided t-test was used to determine differences in response. *Results.* Perifosine induced significant cytotoxicity and inhibition of DNA synthesis in WM cell lines with an IC50 of 5-20uM in all cell lines tested. Similar effects were demonstrated in 3 primary CD19+ WM cells obtained from patients' bone marrow. Cell cycle analysis demonstrated G1 arrest at 24hrs. The effects of perifosine were significant even in the presence of BM stromal cells that induce resistance. Perifosine did not induce cytotoxicity in healthy donor peripheral blood mononuclear cells indicating no toxicity on normal cells. In addition, low doses of perifosine (5uM) induced a decrease in IgM secretion in WM cells at 12 hrs of incubation. *Conclusion.* Perifosine has significant antitumor activity in WM *in vitro*. *in vivo* studies are ongoing. These results provide the framework to test perifosine as a new therapeutic agent in patients with WM. Supported in part by the Leukemia and Lymphoma Society, the Lymphoma Research Foundation and an American Society of Hematology Scholar Award.

0614

PRECLINICAL PHARMACOLOGY AND *IN VITRO* CHARACTERIZATION OF PR-171, A NOVEL INHIBITOR OF THE 20S PROTEASOME

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Background. Clinical studies using the boronic acid-based proteasome inhibitor bortezomib (Velcade™) have validated the proteasome as a therapeutic intervention point for the treatment of multiple myeloma and non-Hodgkin's Lymphoma. Despite encouraging clinical responses, significant hematologic and neurotoxicities have restricted the intensity of bortezomib dosing. *Aims.* To assess the *in vitro* and *in vivo* activity of PR-171, a novel peptide epoxyketone inhibitor of the proteasome. *Methods.* Proteasome activity was measured *in vitro* using purified enzyme and lysates of tumor cells treated with PR-171. Cytotoxicity against tumor cell lines was measured following continuous and pulsatile exposure to PR-171. Pharmacokinetics, pharmacodynamics and toxicity were determined in rats and monkeys following IV bolus administration of PR-171 and anti-tumor activity was assessed in immunocompromised mice bearing established human tumor xenografts. *Results.* PR-171 irreversibly inhibits the chymotryptic subunit of the 20S proteasome and is >50-fold selective over the other proteasome catalytic activities. PR-171 potency for this proteasomal subunit in tumor cells correlates with its cytotoxic potential resulting in IC50 values <10 nM for both activities in multiple cell lines. Furthermore, the *in vitro* cytotoxic activity of PR-171 is retained in tumor cells resistant bortezomib. In experimental animals, PR-171 administration results in a prolonged dose-dependent inhibition of the 20S proteasome in all tissues examined with the exception of brain. Single doses resulting in greater than 90% inhibition of proteasome activity in blood and most tissues are well tolerated in rodents and monkeys. Recovery of proteasome activity following exposure to this irreversible inhibitor is dependent upon synthesis of new proteasome subunits and occurs with a t1/2 of ~ 24 hr in nucleated cells. The safety of PR-171 has been assessed in rodents using multiple dosing schedules including weekly, biweekly and daily administration. In rats and monkeys, daily administration of PR-171 at doses that resulted in >80% inhibition of proteasome activity were well tolerated. Recovery of proteasome activity in tissues following repeated daily administrations was unchanged from that seen after a single dose. At the MTD, transient thrombocytopenia was noted but no other hemotalogic abnormalities were present. In human tumor xenograft models, PR-171 is able to mediate an anti-tumor response against solid and hematologic tumors that is both dose and schedule dependent. PR-171 is active in multiple lymphoma models including a model for Burkitt's lymphoma. *Summary.* These studies demonstrate the tolerability, anti-tumor activity and dosing flexibility of PR-171 and provide validation for the clinical testing of PR-171 in the treatment of hematologic malignancies utilizing dose intensive schedules.

0615

ANALYSIS OF DELTA AND Γ T-CELL RECEPTOR GENES IN MYCOSIS FUNGOIDES AND SZARY SYNDROME

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Background. Demonstration of a dominant T-cell clone in Mycosis Fungoides (MF) and Sézary Syndrome (SS) is usually made with β and γ probes and rarely with Δ probes. *Aims.* We studied T-cell clonality for TCR Δ and γ chain gene in the peripheral blood (PB), bone marrow (BM) samples and cutaneous lesions of 14 patients with early-stage MF and 10 patients with advanced-stage MF and SS. *Methods.* A total of sixty four specimens were analysed: 11 skin biopsies and 14 PB cells from patients with early-stage MF; 4 skin biopsies, 28 PB cells and 7 BM samples from patients with advanced-stage MF and SS. PCR for TCR Δ gene rearrangement analysis was performed according to Hettinger *et al.* (1998); following PCR, amplified products were visualised by high resolution electrophoresis on automated DNA sequencer ABI PRISM 310. PCR amplification for TCR γ gene was performed as previously reported (Ashton-Key *et al.*, 1997); duplicated amplification products were visualised by 10% polyacrylamide gel electrophoresis (PAGE). *Results.* In patients with early-stage MF, monoclonality for TCR Δ gene was detected in skin in 91% of the cases, in PB in 36% and both in skin and PB in 27%. TCR Δ gene rearrangements for VΔ3-JΔ1 and VΔ1-JΔ1 were observed, respectively, in 64% and 27% of cases; in 2 patients VΔ3-JΔ1 monoclonal pattern was also associated with a VΔ2-JΔ1 mono/oligoclonal expression. Dominant clonal TCR γ gene rearrangements were detected in skin in 85% of the cases, in PB in 50% and both in skin and PB in 22%. In 10 patients with advanced-stage MF and Sézary syndrome, TCR Δ γgene analysis detected T-cell clones in the PB in 90% of cases; two molecular patterns were observed: VΔ3-JΔ1 (80%) and VΔ1-JΔ1 (20%). Identical molecular patterns were detected on 4 patients having skin biopsies simultaneously obtained with PB specimens. Moreover we identified some patients with stable clonal pattern in multiple sequential PB specimens (2 clinical responses and 3 progressive diseases during the follow-up period) and others with both persistent and variable clonal pattern (1 clinical response and 2 progressive diseases). Identical T-lymphocyte clones were also detected in 7 cases in BM samples and PB specimens, where conventional light microscopic examination of BM trephine biopsy had failed in detecting occulte disease.The percentage of TCR γ rearrangements in advanced-stage MF and SS well correlated with TCR Δ results. *Summary/Conclusion.* 1) Both PCR techniques for TCR Δ and TCR γ gene showed reproducible results for detecting identical circulat-

ing and cutaneous T-cell clones in MF/SS 2) In comparison with PAGE, the TCR Δ PCR assay using capillary electrophoresis allowed a more precise assessment of the molecular pattern at diagnosis and during follow-up; all TCR Δ rearrangements were VΔ3-JΔ1 and VΔ1-JΔ1, often integrated in their mono-oligoclonal expression. Our finding of a restricted pattern of rearrangements support the suggestion of a specific antigen in MF/SS; moreover it is intriguing that some Authors consider the clonotypic TCR as a source of tumor-specific antigens and a possible target for recognition by CD8+ CTLs.

0616

IMMUNOGLOBULIN V GENES IN WALDENSTROMS MACROGLOBULINEMIA REVEAL ECTOPIC SOMATIC MUTATIONS BUT NO GLYCOSYLATION MOTIFS

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Background and Aims. In Waldenstrom's macroglobulinemia (WM), current evidence from Ig VH gene analysis indicates heterogeneous disease origins. Most cases appear to derive from post-follicular B-cells with somatically mutated (MUT) VH genes, but a few WM display unmutated VH genes, consistent with pre-germinal center (GC), naïve B-cell origins. In MUT WM, expression of CD27, regarded as a marker of post-GC memory B-cells also appears to be variable, with tumor cells apparently identifiable in both CD27+ve and CD27-ve fractions, raising further questions about the cell of origin. Here, we re-evaluate V gene mutational features in MUT WM. *Results and Discussion.* In MUT WM cases, we used single cell analysis to reveal intraclonal variability in CD27 expression. Failure to express CD27 could be disease-associated or may reveal an unusual cell of origin. Interestingly, both CD27+ and CD27- tumor cells unexpectedly showed evidence for low level ongoing somatic mutation in V(D)J-Cmu sequences. These data suggest that ectopic, continuing mutational events occur in WM in the bone marrow. To probe this further, we examined the expression of activation induced cytidine deaminase (AID), a pre-requisite for mutational activity. AID transcripts were identifiable in single CD27+ and CD27- cells, albeit at a low frequency. A striking feature of lymphoma cells that undergo continual somatic mutation, and remain in the GC site, is their ability to generate novel glycosylation motifs via mutated nucleotides in V genes. These are functional and in follicular lymphoma appear mandatory, suggesting a role in tumor-stroma interactions. Given that localised mutations can be identified in MUT WM, we examined a series of these cases for such sites, using paired VH and VL analysis in 14 cases. In these, and in a further 7 WM VH genes, the incidence of glycosylation sites was at a low, background level. Somatic mutations in MUT WM therefore appear not to lead to acquisition of novel glycosylation sites. Such modifications are also absent in MUT chronic lymphocytic leukemia (CLL). These findings indicate no relationship between WM and GC lymphoma tumors. Instead, location in bone marrow and heterogeneity in somatic mutational activity point to a closer similarity to CLL of tumor behaviour at this site.

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0617

IDENTIFICATION OF POTENTIAL PROGNOSTIC MICRORNAS IN DIFFUSE LARGE B CELL LYMPHOMA BY EXPRESSION PROFILING

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Background. MicroRNAs (miRNAs) are a recently discovered class of naturally occurring short non-coding RNA molecules that regulate gene expression through translational repression. They have been shown to play a critical role in many biological functions and there is emerging evidence to suggest that dysfunctional expression of miRNAs is a common feature of malignancy. The identity of lymphoma-associated miRNAs however remains poorly defined. Diffuse large B cell lymphoma (DLBCL) is an aggressive disease accounting for nearly 40% of all lymphoid tumours. DLBCL is characterized by marked clinical and pathological heterogeneity that is reflected at the molecular level. DLBCL can be divided into at least two prognostically distinct molecular subtypes by gene expression profiling; those that are germinal centre B cell like (GCB) and those that are activated B cell like (ABC). It is not known whether similar heterogeneity is also present at the miRNA level. *Results.* We used microarrays to show that the miRNA expression profiles of ABC (OCI-Ly3 and OCI-Ly10) and GCB (SUD-HL4, SUD-HL6 and SUD-HL10) prototype DLBCL cells were distinct, and that expression profiles of all cell lines were distinct from normal lymphocyte populations. We validated the microarray data by RNase-protection assay and identified miRNAs expressed exclusively in either ABC (miR-221, miR-222, miR-21 and miR-155) or GCB (miR-342 and miR-181a) cell lines. Using RNase-protection assays we showed that miR-181a was highly expressed in peripheral blood B cells but less so in T cells, whilst the converse was true for miR-342. Both miR-342 and miR-181a were expressed in naïve but not GC or memory B cells. miR-21 and miR-155 were not expressed in lymphocyte populations and miR-221 was expressed but showed no differential expression between populations. Although clearly heterogeneously expressed, there was no association between the pattern of expression of these miRNAs and ABC/GCB immunophenotype in twenty-eight cases of DLBCL we examined by RNase-protection assay. Interestingly, miRNA expression patterns were linked with clinical characteristics of the cases. High miR-181a expression was associated with patients that had undergone high grade transformation from follicular lymphoma ($p<0.01$) and miR-221 expression with the presence of extranodal disease ($p<0.05$). Moreover, patients with high levels of miR-155 expression had shorter relapse-free survival times (RFS) ($p=0.01$), whilst those that expressed miR-342 had longer RFS times ($p=0.05$). *Summary.* These results raise the possibility that miRNAs could be useful molecular diagnostic and prognostic indicators for this heterogenous disease and probably for haematological malignancies in general.

0618

EVIDENCE FOR A PATHOPHYSIOLOGICAL ROLE OF CYSTEINYL LEUKOTRIENES IN HODGKIN LYMPHOMA

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Background. Classical Hodgkin Lymphoma (cHL) is a predominantly B lymphocyte-derived neoplasia characterized by a minority of malignant cells, the so-called Hodgkin- and Reed Sternberg (H-RS) cells, surrounded by inflammatory cells such as eosinophils and mast cells. In contrast to other tumours, the malignant H-RS cells constitute only a few percent of the total cells in the affected tissue. Therefore, it is generally believed that various compounds released by H-RS cells are of great importance in the pathophysiology of cHL disease. *Aim.* To characterise the expression of cysteinyl leukotriene 1 receptors (CysLT1R) in cHL. *Methods and Results.* We have identified functional CysLT1R in a HL cell line as shown by increased intracellular calcium release upon leukotriene (LT) D4 stimulation (100-500 nM). This response was completely blocked after addition of zafirlukast, a specific CysLT1R antagonist. Immunohistochemical studies of paraffin embedded cHL tissue showed H-RS cells positive for CysLT1R in 12 of 16 cHL tumours. The HL cell line was cultured in the presence of LTD4 (100 nM) to investigate the effects of CysLT signaling in H-RS cells. Real-time RT-PCR analysis showed up-regulation of TNF-α, interleukin (IL)-6, IL-8 and IL-13 mRNA after stimulation with LTD4. Furthermore, the effects of LTD4 on cytokine protein secretion by the HL cells were studied by flow cytometry. The results showed a markedly increased secretion of TNF-α, IL-6 and IL-8 upon LTD4 stimulation. *Conclusion.* Since H-RS cells are surrounded by cysteinyl leukotriene producing cells (eosinophils and mast cells), these results indicate that CysLT signaling could be of importance in the pathogenesis of cHL by contributing to the disturbed cytokine features of this tumour.

This study was supported by the Swedish Cancer Society

0619

HUMAX-CD20, A NOVEL FULLY HUMAN ANTI-CD20 MONOCLONAL ANTIBODY: BIOLOGICAL PROPERTIES

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Background. Monoclonal antibodies (mAb) play a crucial role in host immune defense and are increasingly recognized as effective therapeu-

tics in a range of conditions. The CD20 molecule expressed on B cells is the best validated therapeutic target for B-malignancies. Using human Ig transgenic mice, we have generated a panel of fully human CD20 mAb (HuMab) directed against the human CD20 molecule. Characterization of these antibodies revealed that two types of CD20 specific antibodies exist: type I CD20 mAb, exhibiting similar characteristics to the chimeric mAb rituximab, and type II CD20 mAb, being functionally comparable to the murine anti-CD20 mAb Tositumomab (B1). *Aim.* The biological property of one of the type 1 mAb, HuMax-CD20, has been evaluated performing *in vitro* and *in vivo* experiments. *Methods and Results. In vitro* experiments showed that HuMax-CD20 has an unusually slow off-rate, and induced rapid translocation of CD20 into lipid rafts. Analysis of its CDC potential showed that HuMax-CD20 recruited C1q to the surface of CD20-positive cells and mediated tumor cell lysis via activation of the classical pathway of complement. Importantly, HuMax-CD20 was exceptionally active in CDC in the presence of human plasma or whole blood, being able to lyse a range of rituximab-resistant targets cells, such as CD20-low expressing CLL. This CDC potency appeared to be related to the unusually slow off-rate of these human antibodies. Our current data on epitope mapping indicated that the CDC potency might be influenced by the region of the CD20 recognized by HuMax-CD20. Binding by rituximab and mouse CD20 mAb, had an absolute requirement for alanine, and proline at positions 170, and 172, respectively, within the large extracellular loop of CD20. Epitope mapping studies, using both mutagenesis studies and overlapping 15-mer peptides of the extracellular loops of CD20, revealed a novel binding site required for binding of HuMax-CD20. The HuMax-CD20 binding epitope is located amino terminally of the binding site for rituximab and is also located in the extracellular loop of CD20. Thus, while off-rate may influence biological activity of mAb, the most critical factor determining CDC potency by CD20 mAb, appears to be the region within the target molecule they recognize. *In vivo* experiments showed that HuMax-CD20 increased survival in a SCID xenograft model. I.v. infusion of HuMax-CD20 in cynomolgus monkeys lead to a profound, long lasting B cell depletion, which recovered after the last dose. HuMax-CD20 has been selected for further clinical development. HuMax-CD20 is currently in phase I/II trials for follicular lymphoma, and B-CLL, and in a phase II trial in Rheumatoid Arthritis. *Conclusion.* These results indicate that HuMax-CD20 holds considerable promise for improved clinical activity and may represent an attractive candidate to treat patients with B-cell malignancies and autoimmune disease.

0620

THE INFLUENCE OF INTERLEUKIN- 10 PROMOTER GENE POLYMORPHISM ON THE OCCURRENCE OF NON HODGKIN LYMPHOMA IN SUBJECTS INFECTED WITH HEPATITIS C VIRUS

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Background. HCV along with chronic liver disease is also considered a causative agent of other clinical pathological conditions which testify the possible direct pathogenic role of the virus in several different cell types including hepatocytes and leukocytes. Prevalence of HCV is significantly higher also in patients suffering with NHL and, all around the world, it was confirmed except for patients studied in North Europe and some areas of North America. In Italy, different groups showed prevalence ranging from 15 to 30%. *Aims.* The goal of this paper is to establish if a polymorphic gene encoding for cytokine could be a predisposing factor for this condition. *Methods.* To do this, we analyzed the distribution of the polymorphism of IL-10-1082 G/A in 63 patients, not infected with HCV, with Non Hodgkin Lymphoma (NHL/HCV-) and in 50 patients, infected with HCV, with chronic active hepatitis, with Non Hodgkin Lymphoma, (NHL/HCV+). *Results.* In this study, for the first time we show that irregardless of age, sex, virus genotype and/ or severity of chronic liver disease a significant prevalence of IL-10-1082 GG genotype seems to influence the occurrence of NHL in HCV infected patients. In fact the distribution of the IL-10-1082 G/A polymorphism was different between NHL/HCV+ and NHL/HCV- patients (p=0.028). The frequency of the IL-10'1082 G allele (p=0.019) and the frequency of the IL-10 -1082 GG genotype against overall genotypes (IL-10-1082 GA/AA) were significantly higher in NHL/HCV+ patients as compared with NHL/HCV- patients (p=0.014).

0621

ALEMTUZUMAB FOR THE TREATMENT OF SEZARY SYNDROME: CLINICAL AND IMMUNOLOGIC FINDINGS IN 10 PATIENTS

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Background. Sézary syndrome (SS) is a cutaneous T-cell lymphoma, for which chemotherapy resistance is a major obstacle for effective therapy. Alemtuzumab (Campath®, MabCampath®) has been shown to be effective for the treatment of SS, but often with severe hematologic toxicity and an increased risk of infection. *Aims.* To determine if an altered, patient-specific regimen of subcutaneous (SC) alemtuzumab can induce hematologic, immunologic, and clinical responses similar to that achieved in response to the standard regimen in patients with SS, while reducing treatment-related toxicity. *Methods.* Alemtuzumab was administered as follows: 3 patients received 3 mg on Day 1, 10 mg on Day 3, and 15 mg on alternating days thereafter; 6 patients received 3 mg on Day 1, and 10 mg on alternating days thereafter; 1 patient received 1 mg on Day 1, 3 mg on Days 3 and 5, and 6 mg on Day 7. Alemtuzumab was administered until the number of circulating Sézary cells was <1,000/mm^3. Peripheral blood lymphocytes were monitored for Sézary cells by 3- or 4-color flow cytometry each day before alemtuzumab treatment until there were <1,000 cells/mm^3. Clinical and immunological responses were measured every 2 weeks for the first 2 months, and monthly thereafter. *Results.* Ten patients (median age 72 years [range, 48-82]) with SS were enrolled in this study; 8 patients with relapsed/refractory disease after ≥1 prior therapies, and 2 patients with newly diagnosed disease with a high peripheral blood cell count. Treatment with alemtuzumab resulted in a median decrease of 96% (range, 78%-99%) in Sézary cells, with 9/10 patients having <1,000/mm^3 in 4-13 days after the start of treatment. Clinical responses were achieved in 9/10 patients (1 CR, 8 PR), and 1 patient had SD after 4 weeks of therapy. Median survival was 13 months (range, 5-32 months); 2 patients died due to progressive disease, and 1 from infectious complications. After a median follow-up of 14 months (range, 5-32 months), 2 patients relapsed after 4 and 7 months, respectively, while 7 patients remain in remission. Hematologic toxicity was mild; 1 patient developed thrombocytopenia grade 2'3 and anemia grade 1. Cytomegalovirus was detected in 3 patients during follow-up, which resolved after intravenous (IV) ganciclovir treatment. Three patients developed sepsis that resolved with IV antibiotics in 2/3 patients, while the other patient died from infectious complications. Infectious complications occurred in all 4 patients who were treated with alemtuzumab 15 mg, but did not occur in any patients treated with the 10-mg dose (p=0.0048). A significant decrease in the absolute values of the normal CD3+CD4+ cells was observed immediately after the first cycle (p=0.002); however, the percentage of NK and CD3+CD8+ cells increased significantly after the first cycle (p=0.006 and p=0.002, respectively). *Summary/Conclusion.* Subcutaneous alemtuzumab, administered at a schedule that is based on the number of circulating Sézary cells, was well tolerated and resulted in a high response rate with durable remissions in patients with Sézary syndrome.

0622

LENALIDOMIDE INHIBITS PROLIFERATION OF THE CHROMOSOME 5 MUTANT HEMATOPOIETIC TUMOR CELL LINE NAMALWA CSN.70 AND INTERFERES WITH RECEPTOR SIGNALING THROUGH THE SHC/GRB2/GAB1/AKT PATHWAY

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Background. Lenalidomide (Revlimid®) has recently been approved for the treatment of a subset of myelodysplastic syndromes (MDS) and is currently being evaluated as treatment for a broad range of other hematology and oncology conditions, including multiple myeloma, chronic lymphocytic leukemia and solid tumor cancers. Lenalidomide efficacy has been reported in clinical trials of MDS patients with a 5q- cytogenetic abnormality, with or without other cytogenetic abnormalities. *Aims.* The present study examines the molecular mechanism of action of lenalidomide in the chromosome 5 deleted Burkitt's Lymphoma tumor cell line, Namalwa CSN.70. *Methods.* Karyotype Analysis. Metaphase chromosomes were studied using G-banding techniques. Cell proliferation assay. Cells were incubated in 96-well cell culture plates with compounds for 72 hours and assayed by 3H-thymidine incorporation. IC50s were calculated by nonlinear regression analyses with GraphPad Prism. Immunoblot and immunoprecipitation. Cells were compound treated then stimulated with Epo (R&D Systems). Lysates were immuno-

precipated with Epo Receptor or Grb2 Abs, probed with phospho-Akt, phospho-Shc, phospho-Gab1 or IRF-1 Abs and analyzed on a Storm 860 Imager using ImageQuant software (Molecular Dynamics). Cell cycle analysis. Cells were compound treated for 72 hours, propidium iodide stained using CycleTEST PLUS (Becton Dickinson) and analyzed by a FACSCalibur flow cytometer using ModFit LT software (Becton Dickinson). Apoptosis analysis. Cells were compound treated for 72 hours, washed with Annexin-V wash buffer, incubated with Annexin-V binding protein and propidium iodide (BD Biosciences) and analyzed using flow cytometry. Luciferase assay. Cells were transfected with AP1- or Egr1-luciferase (Stratagene), compound treated then assayed using luciferase substrate (Promega) and measured using a luminometer (Turner Designs). *Results.* Lenalidomide inhibited proliferation of these cells *in vitro* and induced G0/G1 cell cycle arrest. Receptor signaling pathways utilize multiple tyrosine phosphorylation events and scaffolding adaptor complexes, such as Shc/Grb2/Gab1 adaptor protein complexes to transduce signals through the Akt pathway. Lenalidomide inhibited Akt and Gab1 phosphorylation in stimulated Namalwa cells. Further upstream of Gab1 phosphorylation, lenalidomide had no effect on the Gab1/Grb2 interaction which relies upon a polyproline / SH3 interaction. However, lenalidomide did interfere with phosphorylation of Shc, an adaptor protein which signals between the receptor and Grb2. Lenalidomide's ability to inhibit Shc phosphorylation lead to inhibition of the Shc/Grb2 interaction. The Shc/Grb2 interaction relies upon a phosphotyrosine / SH2 interaction, however, lenalidomide did not directly inhibit this interaction in a cell-free system. Rather, lenalidomide inhibited Gab1 association with the receptor in the intact cells, suggesting that there was disruption of the adaptor protein complex via another mechanism. Lenalidomide also enhanced AP-1 and Egr1 transcriptional activity in Namalwa cells. Interestingly, Egr1 is a zinc-finger transcription factor and tumor suppressor whose gene is located on 5q31.1. *Conclusions.* These studies provide evidence that the mechanism of action of lenalidomide in this chromosome 5 deleted Burkitt's Lymphoma cell line *in vitro* involves inhibition of the Akt signaling pathway as early as inhibition of phosphorylation of the Shc scaffolding protein. In addition, lenalidomide enhances the activity of the Egr1 tumor suppressor, which may explain the special sensitivity of del 5q tumor cells to lenalidomide.

0623

DETERMINATION OF LYTIC PHASE CELLS AND INTEGRATION STATUS OF EPSTEIN-BARR VIRUS INFECTED CELLS LINES

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Epstein-Barr virus (EBV, human herpesvirus type 4) is ubiquitously distributed in all human populations, reaching infection rates of more than 90%. EBV is known to infect B- lymphocytes and mucosal epithelium cells and to establish latent or productive infections. The virus is the causative agent of infectious mononucleosis and closely associated with the endemic form of Burkitt lymphoma. EBV has also been associated with various lymphoid and epithelial malignancies, such as Hodgkin, T-cell, and AIDS-related lymphomas, and lymphoepithelioma-like carcinomas of several organs. *In vitro*, B- lymphocytes are transformed by EBV into permanent lymphoblastoid cell lines (B-LCL). Active EBV particles contain linear double-stranded genomes, which are circularized intracellularly. The episomes persist in the nucleus and can be integrated into the eukaryotic genomes. We determined the EBV infection status of a panel of 421 primate cell lines by PCR (417 human, 4 monkey). 39 cell lines (38 human, 1 monkey) contained EBV sequences. All cell lines were established from B-lineage primary cells. No cell lines from other tissues were found to be EBV+. To investigate the number and the integration status of the EBV genomes and the ratio of lytic phase cells in a cell culture population, we established a fluorescence *in situ* hybridization (FISH) method on cytospin preparations of untreated cells and on metaphase spreads of colcimid treated cells. Hybridization was performed with various Cy3 and Spectrum Red/Green labeled cosmid clones encompassing almost all of the EBV genome. The B95-8 cell line, which was described to produce active EBV particles was used as positive control. The number of EBV copies varied highly among the cell lines. This was demonstrated by FISH and by Southern blotting. The number of episomes was not evenly distributed among the individual cells of a cell culture: a proportion of the cells contained numerous episomes, whereas the highest fraction of the nuclei contained many fewer copies of EBV. At one end of the spectrum is the cell line DOHH-2 with a few cells exhibiting ca. 50 copies of EBV, whereas the rest of the cells are EBV-negative by FISH. Some cell lines show a small fraction of lytic phase EBV infected cells which could be increased by treatment with the phorbol ester TPA. The appearance of lytic phase cells corresponds to the demonstration of linear EBV bands applying *in situ* lysing gel analysis. On the contrary, for most of the cell lines with lytic phase cells, no immediate early protein BZLF1 could be detected by Western blotting. Metaphase FISH revealed that most of the cell lines which harbor EBV sequences contain integrated sequences determined by paired signals as well as episomal DNA. The number of integrated sequences range from 1 (cell line NAMALWA, no episomes; cell line CI-1, several episomes) to about 100 integration sites (cell line RAJI, many episomes). In summary, we could show that FISH can be used to recognize lytic phase cells in cell cultures and to determine the amount of EBV genomes per cell and the integration status of the EBV genome.

0624

UTILITY OF FLOW CYTOMETRIC SCREENING ON FINE-NEEDLE ASPIRATES IN THE DIAGNOSIS OF CHRONIC LYMPHOPROLIFERATIVE DISORDERS

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Background. At present, diagnosis of chronic lymphoproliferative disorders (CLPD) on lymphoid tissue samples is performed by a combination of different techniques; histopathological and immunohistochemical analyses are considered the reference approaches. To date, large studies evaluating in a prospective way the reliability of flow cytometry (FCM) for the screening of CLPD by comparison with both cytologic and histologic diagnoses in the same fine-needle aspiration (FNA) sample remains scarce. *Aim.* To establish the utility of FCM for the screening of CLPD from lymphoid tissue samples obtained by FNA. *Materials and Methods.* A total of 263 correlative samples obtained by FNA were stained with CD8-FITC/-PE/CD4-CD19-PCy5/CD3-APC (screening tube). Reference microbeads (Perfect Count) were added, to calculate the absolute numbers of cells/sample. Data acquisition was performed on a FACSCalibur flow cytometer. Data obtained by FCM were compared with those obtained by cytological and histological techniques. For the discrepant cases, the final diagnosis was based on either molecular/genetic or histological analyses. In the statistical comparisons, p values ≥0.05 were considered to be statistically significant. *Results.* In 228 samples (87%), FMC was concordant with the diagnosis made by cytology and histology.

Table 1. Discrepant cases when FCM, cytology and/or histology techniques were compared (n=29).

Case N.	Age/Sex	FCM	Cytology Diagnosis	Histopathologic	PCR/SB	Final Diagnosis
1	15/F	RP	Non conclusive	T-NHL	POLYCLONAL	RP
2	74/M	RP	ST vs NHL	B-NHL	POLYCLONAL	RP
3	78/F	RP vs Non conclusive	PCD	-	-	PCD
4	64/F	RP	PCD	-	-	PCD
5	48/F	RP	Non conclusive	Clonal plasma cells	-	PCD
6	85/F	RP	NHL	B-NHL	POLYCLONAL	RP
7	73/F	B-NHL	NHL	HL	T(14,18) HL/NHL	HL/B-NHL
8	88/F	B-NHL	RP	-	-	B-NHL
9	71/M	B-NHL+ST	ST	ST	FISH: Typical B-CLL alterations negative ST abnormalities	ST/B-NHL
10	75/F	T-NHL	NHL	B-NHL	TCRB+	T-NHL
11	73/F	T-NHL	HL	HL	TCRB+	T-NHL/HL
12	36/M	T-NHL	RP	T-NHL	TCRB+	T-NHL
13	48/M	PCD	Low cellularity			
14	80/M	RP vs ST	Non conclusive	B-NHL		
15	44/F	DCL	GRANULOCYTIC SARCOMA	GRANULOCYTIC SARCOMA	-	DCL
16	24/M	RP	Low cellularity	RP	-	RP
17	73/M	RP	Low cellularity	-	-	RP
18	72/M	RP	Low cellularity	-	-	SARCOMA
19	?/M	RP	Low cellularity	-	-	-
20	31/M	RP	Low cellularity	-	-	HL/B-NHL
21	72/M	ST+B-NHL	Low cellularity	-	-	B-CLL+B-NHL
22	44/M	AML	Low cellularity	-	-	AML
23	95/M	FB contamination	ST	-	-	-
24	62/M	FB contamination	Low cellularity	-	-	-
25	30/F	FB contamination	Low cellularity	-	-	HL/B-NHL
26	15/M	FB contamination	FB contamination	-	-	RP
27	73/M	Low cellularity	Low cellularity	B-NHL	-	B-NHL
28	75/F	Non conclusive	Non conclusive	B-NHL	-	B-NHL
29	79/F	Non conclusive	Non conclusive	AML	-	AML

F:female; M: male; NHL: non hodgkin Lymphoma; PCD: Plasma cell Discrasia; RP: Reactive/process; ST: solid tumor; HL: Hodgkin's lymphoma; DCL: Dentric cell leukemia; AML: Acute myeloid leukemia; -: Not analyzed; PB: Peripheral blood.

In 69 out of the 228 samples (30%), the final diagnosis was compatible with B-non-Hodgkin Lymphoma (B-NLH) (53 cases sIgkappa+, 15 sIgLambda+ and one case sIgGamma), 113 cases (49%) were considered as reactive processes (RP; n=97) or Hodgkin disease (HD; n=16, confirmed by histology) and in 27 (12%) infiltration by non-hematopoietic cells was detected (solid tumor -ST- by histology). In the remaining 19 cases (8%), the diagnoses corresponded to: plasmacytoma (n=7), T-cell CLPD (n=6), T-acute lymphoblastic leukemia (T-ALL; n=5) and acute myeloid leukemia (AML; n=1). Discrepant samples (n=35) corresponding to 29 cases are shown in Table 1a and 1b. In 14 out of these 29 cases (48%) the final diagnosis was concordant with that provided by FCM, while in 3 (10%) cytology gave the correct diagnosis; in the remaining 12 cases (41%) the diagnosis was not conclusive, mainly due to low cellularity or peripheral blood contamination. The sensitivity and specificity of FCM in diagnosing the different CLPD ranged between 94-100% and 88-100%, respectively. Overall, in B-NHL the percentage of clonal B-cells was significantly higher than in the other groups (59±25% vs 0%), all these cases showed an imbalance in the k-λ ratio. Also, a significantly higher percentage of T-cells was found in T-NHL as compared to the other groups -except AML- (59±24% vs 36±25%). In ST, a variable infiltration by non-hematopoietic cells was found (38±34%); the diagnosis of plasmacytoma, dendritic cell (DC) neoplasia, T-ALL and AML was based on the identification of increased numbers of plasma cells (55±38%), DC precursors (99%), T-lineage blast-cells (57±41%) and myeloid blast-cells (96%), respectively. Of note, the diagnosis was established from relatively low numbers of cells (1.9±3.6 ($\times 10^6$)). *Conclusion.* screening by FCM of lymphoid tissue samples obtained by FNA is a precise, fast and cheap tool to be used for the diagnosis of CLPD, requiring a relatively low numbers of cells, although it is not useful for the diagnosis of HD.

0625

ANAPLASTIC LARGE-CELL LYMPHOMA A RETROSPECTIVE MULTICENTRIC CLINICAL AND PATHOLOGICAL STUDY

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Background. Anaplastic Large Cell Lymphoma (ALCL), a rare Non-Hodgkin's Lymphoma (NHL), with less than 5% incidence, has a predilection for younger patients, disseminated disease at presentation, and extranodal determination. A standardized treatment is not yet available. *Aims.* The aims of our study are to analyze the clinical and pathological correlation of 45 cases of T and null ALCL. *Methods.* This is a multicentric retrospective clinical and pathological study of 45 cases diagnosed in ours hospitals between 1997-2004; a lot of monoclonal antibodies were utilized for phenotypic evaluation by immunohistochemical techniques. *Results.* The incidence of ALCL was low (only 2.13% from all NHL), but they represented 1/4 of T-NHL. Although it was a large diversity of pathological aspects, about 80% of the cases were classified as common type. T-phenotype, especially T-suppressor, was more frequent. The malignant cells constantly expressed CD30; 88% of cases were EMA positive, and 56.5% ALK positive. The bimodal age distribution of patients was evident in ALK positive cases without correlation with the T/null phenotype and the male predominance more evident in cases with T phenotype and respectively ALK positive. Approximately 2/3 of patients were diagnosed in advanced stages of disease, 82% presented B symptoms at presentation. 42% presented extranodal disease especially cutaneous and bone involvement. Because of the young age and good performance index, most patients were classified in accordance with IPI in low and intermediary low risk. Even the therapeutically approach was diverse, in most cases were used CHOP protocols; autologous bone marrow transplantation was performed only in two cases, with complete remission in one case. The major predictive factors correlated with a favorable prognosis were IPI score ≤ 2 and ALK expression by malignant cells. *Conclusions.* In the majority of cases the ALCL diagnosis is rather difficult; the young age predominance and the aggressively of the disease justify the need for a new therapeutically targets correlated with the phenotypic expression of the malignant cells.

0626

EXPRESSION OF CD52 IN T-CELL AND NK/T-CELL LYMPHOMAS

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Background. CD52 antigen, also known as CAMPATH-1, is a heavily glycosylated small peptide linked to the surface membrane. It is expressed in high density by lymphocytes, monocytes/macrophages, eosinophils, thymocytes and macrophages, but is absent in granulocytes, platelets, red cells, and bone marrow stem cells. CAMPATH-1H or Alemtuzumab is a genetically reshaped human IgG1 monoclonal antibody against CD52. It has been shown to be effective in T-cell malignancies, particularly in T-cell prolymphocytic leukemia and cutaneous T-cell lymphoma (TCL). It is also active in those with peripheral TCL (PTCL) who were refractory or had been heavily pretreated with conventional chemotherapy, although it is associated with significant hematologic toxicity and infectious complications. To date, there have been very limited studies focusing on the expression of CD52 in TCL. *Aims.* To investigate CD52 expression in TCLs. *Methods.* Immunohistochemical study using anti-CD52 (MCA1642, Serotec, Oxford, U.K.) in 96 cases of TCLs. Moderate to strong staining in _ 20% of tumor cells is considered positive. *Results.* CD52 is expressed in 35 (36%) cases including 10/14 (71%) angioimmunoblastic T-cell lymphomas (AITLs), 15/34 (44%) unspecified PTCLs, 4/17 (24%) NK/T-cell lymphomas, 3/18 (17%) anaplastic large cell lymphomas, 1/10 (10%) T-lymphoblastic lymphomas, 1/1 panniculitic TCL, 1/1 adult T-cell leukemia/lymphoma, but not in one hepatosplenic TCL. *Summary/Conclusions.* Our results show that one third of T-cell malignancies express CD52 with various frequency among various subtypes and suggest that AITL and unspecified PTCL are better candidates than other subtypes for CAMPATH-1H treatment. It might be advisable to perform CD52 immunostaining before starting CAMPATH-1H treatment.

0627

SPLENIC MARGINAL-ZONE LYMPHOMA: ONE OR MORE ENTITIES? A HISTOLOGICAL, IMMUNOHISTOCHEMICAL AND MOLECULAR STUDY OF 42 CASES

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The histogenesis of splenic marginal zone lymphoma (SMZL) is currently unknown. We conducted a detailed histological, imunohistochemical and genotypic analysis in a series of 42 SMZL cases, diagnosed on splenectomy specimens after established WHO criteria. A broad spectrum of monoclonal antibodies was used in order to exclude other small B cell malignancies mimicking SMZL in the spleen [CLL, hairy cell leukemia (HCL), mantle cell lymphoma, follicular lymphoma, lymphoplasmacytic lymphoma]. The aim of our study was to correlate phenotypic and molecular findings (IG heavy/light chain repertoire and somatic mutations) so as to gain insight into SMZL immunopathogenesis. A predominantly nodular growth pattern was observed in 24 cases; the remainder showed predominantly (11/42) or exclusively (7/42) diffuse infiltration. Twenty-one cases showed the 'classical' biphasic appearance. The remaining cases (21/42) were monomorphous: 13/21 cases exhibited marginal-zone morphology, while 8/21 cases were composed predominantly of small cells. Five cases demonstrated plasmacytic differentiation. CD21/CD35 were expressed by, respectively, 12/42 and 17/38 cases, generally in a concordant fashion. Residual FDC meshworks were detected by CD21 and/or CD35 staining in all cases. DBA.44, traditionally known as a marker of HCL and normal mantle cells, was detected in 24/42 cases. Seventeen out of 37 cases were SIgD-positive; 12/22 cases expressed SIgM/SIgD, 7/22 expressed SIgM, while one case expressed SIgD only; 5/36 cases were SIgG-positive. CD27 staining was observed in 22/35 cases; 8/19 CD27-positive cases also expressed SIgD; 3/5 CD27-negative cases were SIgD-positive. CD5 was detected in 4/42 cases. Forty IGHV-D-J rearrangements were amplified in 34/42 cases. Among six cases with double rearrangements, three carried double in-frame rearrangements. The most frequent IGHV gene was IGHV4-34 followed by IGHV1-2, IGHV1-18, and IGHV3-30. Two IGHV1-69/IGHD3-16/IGHJ3 rearrangements with identical HCDR3 sequences were identified; a similar, restricted HCDR3 sequence has been reported in CLL cases. Twenty-six IGKV-J rearrangements were amplified in both kappa and lambda expressing cases; IGLV-J rearrangements using six different germline IGLV genes were amplified in 8 lambda-SMZL cases. Using the 98% homology cut-off value, 22/40 IGHV sequences (55%) and 15/34 IGK/LV sequences (44%) were considered as 'mutated'. Nine out of eleven cases (82%) with monomorphous, marginal-zone morphology carried IGHV-mutated genes. In contrast, 4/6 cases (66%) with monomorphous, small-cell morphology carried IGHV-unmutated genes. Six out of seven cases expressing IGHV1 subgroup genes had biphasic morphology. In contrast, 5/9 IGHV3-expressing cases had monomorphous, marginal-zone morphology; 3/9 cases expressing unmutated IGHV4 genes had monomorphous small-cell morphology. All IGHV1-expressing cases were CD21-negative. Most cases (12/19; 63%) with

IGHV-mutated genes were SIgD-negative; in contrast, IGHV-unmutated cases often expressed SIgD (7/12; 58%). CD27 was detected at a similar frequency in either the IGHV-mutated or IGHV-unmutated subgroups (11/16 and 8/12 cases, respectively). Six out of 10 CD27-negative cases carried IGHV-mutated genes; all six CD27-negative/IGHV-mutated cases expressed DBA.44. These results confirm the considerable histological, immunohistochemical and molecular heterogeneity of SMZL and indicate an origin from the diverse resident B cell populations of the normal SMZ. Furthermore, they indicate a role for selective antigenic pressures in the pathogenesis of at least a subset of SMZL cases.

0628

REAL-TIME QUANTITATIVE PCR AND FOUR COLOUR FLOW CYTOMETRY FOR MINIMAL RESIDUAL DISEASE ASSESSMENT OF MANTLE CELL LYMPHOMA

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Background. MCL is an aggressive disease and few patients reach long-term survival. The impact of tumour load estimation and MRD quantification in these patients is unclear. We analysed peripheral blood (PB) and bone marrow (BM) disease levels obtained by both RQ-PCR and FC in 15 patients with MCL, 12 males and 3 females, median age 61 yo (range 53-85), all in stage III or IV, for whom a BM (14 pts) or PB (1 pt) sample was obtained at diagnosis. MCL was diagnosed by lymph node biopsy in all cases. Fourteen patients were treated with therapeutic protocols including anthracyclins in 11, with (N=7) or without (N=4) Rituximab, and clorambucil+prednisone in the remaining 3 cases. Methodology: 31 samples were analysed (27 BM, 4 PB), at time of diagnosis (18/31), after (7/31) or during (4/31) 1st line therapy or during complete remission 1 (2/31). RQ-PCR monitoring was performed through amplification of the BCL1-IGH (Hirt C *et al.,* Blood 2004) in 3/15 pts and using an ASO against the IGH-CDR3 (Verhagen OJHM *et al.,* Leukemia 2000) in 8/15 pts or on both targets in the remaining 4/15 pts. FC was performed using the following pannel: anti CD19, CD20, CD22, CD23, CD10, FMC7, CD5, CD45, K, L. B cells were considerer neoplastic if they were CD19+/CD5+ and exhibited light chain restriction; CD23 and CD10 were always negative. 20000-150000 events were acquired per sample. *Results.* At diagnosis (N=15) FC (sensitivity between 10-4 and 10-5) and RQ-PCR (sensitivity between 2×10^{-4} and 10^{-6}) demonstrated tumour infiltration in all samples analysed. Importantly, in 4 BM samples without histological infiltration, FC and RQ-PCR detected disease levels between 1,2-30,2% and 2,6-19%, respectively. Overall we obtained concordant results between RQ-PCR and FC in 87% of the samples. Discrepant results consisted in 2 samples RQ-PCR-/FC+ and 1 RQ-PCR+/FC- obtained after and during 1st line treatment, respectively. The former can be false positive FC results and the latter can be attributed to the higher sensitivity of the RQ-PCR. Nevertheless, sampling effect can not be excluded. In 5/13 follow-up samples with MRD levels quantified by FC and RQ-PCR (results within the quantitative range), we found a positive correlation between the two techniques: $p<0{,}001$, Pearson correlation r=0,997. There was no significant association between disease levels at diagnosis (n=12) and clinical status at end of 1st line treatment: complete remission (n=5), median quantity (MQ)= 8,79% (0,35-100), partial remission (n=4) MQ=17,57% (3,73-49,13), progression (n=3) MQ=43,75% (2,99-100). *Conclusions.* FC and RQ-PCR tumour burden estimation and MRD quantification correlate well in MCL. Nevertheless, discrepant results can be obtained and both approaches should be used to improve assessment of tumour dissemination. At diagnosis FC and RQ-PCR can detect low levels of disease in patients without histological BM involvement and contribute to a more accurate clinical staging. Longer follow-up studies, based on FC and RQ-PCR are needed to clarify the association between minimal disease levels and clinical outcome.

0629

PHARMACOGENOMIC APPROACH IN WALDENSTRM'S MACROGLOBULINEMIA AND SMALL LYMPHOCYTIC LYMPHOMA PATIENTS: HCNT1 EXPRESSION AS A POSSIBLE PREDICTIVE BIOMARKER OF 2-CHLORO-2'-DEOXYADENOSINE CLINICAL ACTIVITY

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Background. Pharmacogenomic can be used to identify genetic factors that influence drug response, such as single nucleotide polymorphism, RNA splicing, gene expression and transcription. 2-chloro-2'-deoxyadenosine (2-CDA) undergoes complex intracellular metabolism and cell resistance mechanism to 2-CDA is not completely known. *Aim.*In this study we used a pharmacogenomic approach to identify genetic factors that could influence 2-CDA clinical response *Method.* Using ABI PRISM 7000 Real Time platform we amplified seven genes, encoding for equilibrative and concentrative nucleoside transporter (hENT1, hCNT1), deoxycytidine and deoxyguanosine kinase (dCK, dGK), 5'-nucleotidase (5'NT), ribonucleotide reductase catalytic and regulatory (RR1, RR2) subunits, in the bone marrow at diagnosis of 27 patients with Waldenström's Macroglobulinemia or Small Lymphocytic Lymphoma. All patients were treated with 4 courses of combination therapy (2-CDA 0.1 mg/kg for 5 days sc injection and Rituximab at standard schedule). Quantitation was performed using the Delta CT calculation: the value of gene expression was normalised to the calibrator (healthy tissue cells). *Results.* hCNT1; RR2; 5-NT gene expression analysis has shown lower values in patients than in healthy tissue controls. 2 patients who achieved clinical partial remission (PR) presented 100 times lower hCNT1 levels (median 3×10^{-4}, range $0\text{-}6.9\times10^{-4}$) than patients (n=12) in complete remission or very good partial remission (median $2.2*10^{-2}$, range $0.1\text{-}9\times10^{-2}$; $p=0.03$). Three patients showed drug toxicity: 2 of them (with very low hCNT1 levels) interrupted 2-CDA treatment; 1 completed the therapy and now is in follow-up; his basal hCNT1 level was similar to the expression of patients who obtained a complete remission. *Conclusion.* hCNT1 seems to be an important gene involved in 2-CDA clinical activity and its expression may correlate with prognosis. Compared to controls, the low RNA level of hCNT1 exhibited by our patients doesn't seem to be predictive of lack of clinical activity of 2-CDA. However the lower hCNT1 expression detected in patients who achieved only PR could suggest a possible relationship between reduced hCNT1 expression and a diminished clinical activity of 2-CDA. Thus it might be important to explore the possibility of standardizing a quantitative method in order to identify a threshold value which could be predictive of drug resistance.

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LYMPHOMA CELLS MICRODISSECTED FROM PRIMARY SPLENIC LOW GRADE NON-HODGKINS LYMPHOMA BUT NOT NORMAL SPLENIC CELLS CONTAIN HCV GENOME

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The role of HCV infection in the pathogenesis of various types of B cell Non-Hodgkin's lymphomas (B-NHL) has been suggested from epidemiological studies, however molecular mechanisms accounting the neoplastic transformation have been to be determined. To investigate the relationship between HCV infection and B-NHL we studied spleen paraffin-embedded sections of 12 patients with primary splenic B- NHL. 8/12 patients were affected from a high grade B-NHL, 4/12 from a low grade B-NHL. Normal and neoplastic cells were individually microdissected as pure cells populations from tissue sections by using a Laser-assisted microdissection apparatus. The presence of HCV genomes was investigated by RT-PCR in RNA extracted from these two cell populations of each patient. While the HCV genome was found both in neoplastic and in normal cells in all the eight patients with high grade disease, the four low grade B-NHL samples showed the presence of the HCV genome only in lthe ymphoma cells and not in the normal cells. These results suggested a direct role of the virus in the low grade lymphoma transformation. In addition, to investigate the molecular pathways involved in the neoplastic transformation, we evaluated, by quantitative real time PCR, BCL2 expression in the cell populations microdissected from the patients. Interestingly, lymphoma cells isolated from low grade NHL patients showed very low expression of the BCL2 gene thus suggesting that molecular mechanisms of neoplastic transformation in these patients is not BCL-2 mediated as in the other low grade NHLs. In conclusion, our results indicate that at least in low grade NHLs, the HCV might directly involved in the neoplastic transformation, and that the molecular mechanisms may be different respect the other types of low grade NHLs.

Acute myeloid leukemia III

0631

APPLICATION OF EXTENDED COX PROPORTIONAL HAZARD MODELS ON THE DATA SETS OF THE ORIGINAL DATA BASED META-ANALYSIS ON PATIENTS 18 TO 60 YEARS OF AGE WITH CORE-BINDING-FACTOR ACUTE MYELOID LEUKEMIA OF THE GERMAN AML-INTERGROUP

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Background. There is increasing interest to apply survival analysis to data sets with multiple events per subject including on the one hand multiple events of the same type and on the other hand events of different types. In our meta-analysis on CBF-AML (Schlenk *et al.* JCO 2004, 22:3741) we used preponderantly time to first event approaches like relapse free survival. However, such approaches negate the multiplicity of events and especially the influence of treatment after relapse is not taken into account. *Aims.* We performed a re-analyses of the datasets of our meta-analysis using extended Cox proportional hazard models to assess the impact of different treatment strategies during first remission (CR) and after relapse in a unique model. *Methods.* We used three approaches of extended Cox models for the re-analysis: i) the Andersen-Gill (AG) model assuming independence of events in the different time periods *first CR* and *after relapse*, ii) the Prentice-Williams-Peterson (PWP) model assuming that a patients can only be at risk for the second time period *after relapse* until he underwent an event in the first time period *first CR*, iii) the Wei-Lin-Weissfeld (WLW) model allowing a separate underlying hazard for each event. The different models were compared by explained variation using the Brier-Score. The dataset sets were restricted to full-set records for patients achieving a first CR (inv(16) n=158, t(8;21) n=149). The variables in the models were the different treatment strategies [allogeneic transplantation from matched-related donor (ALLO-SCT), allogeneic transplantation from matched-unrelated donor (MUD-SCT), intensive high-dose cytarabine based chemotherapy (CHEMO), autologous transplantation (AUTO-SCT)], trisomy 22 [inv(16)], loss of X or Y [t(8;21)], disease state (CR versus no-CR), dichotomized (>25.4) WBC count [t(8;21)] and dichotomized (>28) platelet count [t(8;21)]. *Results.* The explained variation for the different models in the inv(16)-data set were 0.105 for the AG-model, 0.110 for the PWP-model and 0.038 for the WLW model and in the t(8;21)-data set 0.261 for the AG-model, 0.226 for the PWP-model and 0.161 for the WLW model. The models with highest values in explained variation was the PWP-model in the inv(16)-data set and the AG-model in the t(8;21)-data set. The hazard ratios and 95%-Confidence Intervals (CI) for the PWP-model were ALLO-SCT 0.39 (95%-CI 0.20-0.77), MUD-SCT 0.25 (95%-CI 0.07-0.87), AUTO-SCT 0.63 (95%-CI 0.37-1.06), trisomy 22 0.50 (95%-CI 0.26-0.95) and disease state 0.43 (95%-CI 0.14-1.30) for the inv(16)-data set and for the AG-model were ALLO-SCT 0.95 (95%-CI 0.43-2.1), AUTO-SCT 0.58 (95%-CI 0.24-1.4), MUD-SCT 3.4 (95%-CI 1.2-9.3), loss of Y 1.65 (95%-CI 1.0-2.7), WBC<25.4/nL 0.4 (95%-CI 0.23-0.7), platelets<28 2.3 (95%-CI 1.3-3.8) and disease state 0.2 (95%-CI 0.1-0.43) for the t(8;21)-data set. *Conclusion.* From the statistical point of view extended Cox proportional hazard models can be well applied to datasets of AML patients. From the clinical point of view the beneficial effects of ALLO-SCT in inv(16)-AML raises the question of treatment strategies in first CR and in t(8;21)-AML the very strong impact of the dichotomized WBC and platelet count at diagnosis argues for risk stratification.

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ALL-TRANS RETINOIC ACID AND GEMTUZUMAB OZOGAMICIN AS ADJUNCT TO SALVAGE THERAPY IN PRIMARY REFRACTORY ACUTE MYELOID LEUKEMIA: RESULTS OF CONSECUTIVE PHASE II STUDIES OF THE AMLSG

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Background. Response to first induction therapy is one of the most important prognostic factors in patients with adult myeloid leukemia (AML). Induction of CR or PR is the primary aim in these patients. *Aims.* To evaluate the impact of all-trans retinoic acid (ARTA) and gemtuzumab ozogamicin (GO) given as adjunct to intensive salvage therapy in primary refractory younger patients on clinical outcome. *Methods.* Between 1993 and 2005 255 consecutive patients (median age: 48 yrs, range 16-60 yrs) treated within the AMLHD93 (n=45), AMLHD98A (n=160) and AMLSG 05-04 (n=50, still active) were evaluated. All patients had primary refractory AML after one cycle of ICE (idarubicine, cytarabine, etoposide). The different salvage therapies were as follows: AMLHD93 sequential-HAM (S-HAM) for patients <55 years of age [cytarabine 3g/m^2 bid. days 1,2,8,9, mitoxantrone 10 mg/m^2 days 3,4,10,11], HAM for patients ≥55 years of age [cytarabine 3 g/m^2 bid., days 1-3, mitoxantrone 12mg/m^2 days 2,3]; AMLHD98A: A-HAM [HAM with ATRA 45mg/m^2 days 3-5, 15 mg/m^2 days 6-28]; AMLSG 05-04: GO-A-HAM [A-HAM with gemtuzumab ozogamicin 3 g/m^2 day 1]. *Results.* The distribution of the different salvage therapies was HAM n=21, S-HAM n=22, A-HAM n=118, GO-A-HAM n=53, other n=31 no further therapy n=10. Response according to salvage therapy was as follows:

	GO-A-HAM	*A-HAM*	*S-HAM*	*HAM*
CR	26 (53%)	40 (34%)	5 (23%)	3 (14%)
PR	6 (12%)	33 (28%)	5 (23%)	4 (19%)
RD	15 (31%)	36 (31%) 12 (54%)	12 (57%)	
death	2 (4%)	8 (7%)	0 (0%)	2 (10%)

No CTC-grade 3-5 liver toxicity was seen in patients receiving GO-A-HAM. Logistic regression on the achievement of CR after salvage therapy revealed that regimens containing ATRA (odds ratio 2.0, p=0.05) and GO (odds ratio 2.2 p=0.02) were associated with response. 142 of 255 patients have received stem cell transplantation. One (4%, 95%-CI 0.002-0.18) case of severe veno occlusive disease was seen in 28 so far transplanted patients who have had GO-A-HAM. Median survival was 11.3 months. *Conclusions.* Although retrospective in nature our study suggests that ATRA and GO as adjunct to salvage chemotherapy in primary refractory AML patients improves CR rates.

0633

GENETIC CHARACTERIZATION OF PATIENTS WITH AML-M2. THE JAK2 V617F ACTIVATING MUTATION IS FREQUENTLY FOUND IN CASES WITH NORMAL KARYOTYPE

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The characterization of genetic and molecular aberrations in AML has substantially improved our understanding of the pathogenesis of this disease. The activating V617F mutation of JAK2 has been recently described as a common event in MPD. The same mutation was also found in a small number of patients with either AML or MDS; however, there are few data about the frequency of JAK2 V617F in specific subtypes of AML. We investigated the incidence of this mutation in 10 cell lines and 331 well characterized AML patients, and its association with other factors with a prognosis meaning. V617F genotyping was performed by ARMS as previously described (Jones el al., 2005). All cas-

es tested positive were confirmed by sequencing. Mutations of FLT3 and KIT were also analyzed. A high resolution 50K SNP array (Affimetrix) was used to analyze 19 samples. We found the mutation V617F in 3% of overall AML (10/331): 2 M1 (2/71;2.8%), and 8 M2 (8/84;9.5%), suggesting a correlation with less differentiated leukemias. According to the WHO-classification, the mutated cases were: AML with t(8;21) (1/16;6.2%), AML with inv(16) (1/12;8.3%), AML with multilineage dysplasia MDS (1/28;3.5%), AML without maturation (2/66;3%), and AML with maturation (5/58;8.6%). In 9 of these patients we could analyze other samples in CR or relapse, and we found that the V617F mutation is not a good marker for MRD. Although in 7 cases the mutation disappeared in CR and appeared again when the patient relapses, in one patient we detected V617F in CR, and in other there was no mutation in the relapse. As expected, 22.6% of all AML, and 30% of cases with normal karyotype, had FLT3 mutations. To further characterize the AML-M2 cases, we analyzed KIT (exons 8 and 17) in 64 patients. In 3 cases (4.7%), a mutation affecting codon 816 of KIT was detected: one D816H and 2 D816V, all with t(8;21) (3/12,25%). No exon 8 mutations were found. Interestingly, one patient had mutations in both JAK2 (V617F) and KIT (D816V); and another had both JAK2V617F and ITD-FLT3, showing that in some cases 2 mutations in TK genes could collaborate in the AML transformation. The 50K GeneChip array was used to type DNA from 17 AML samples (8 with JAK2 mutated, and 9 no mutated), and 2 cell lines with JAK2V617F. Five cases (29%), and the 2 cell lines, had regions of uniparental disomy (29%), confirming the importance of this mechanism in AML. The 3 samples with V617F in homozygosis had LOH by UPD in 9p. The data presented in our study confirm the association of KITD816 mutations and t(8;21), whereas mutations of JAK2 and FLT3 were more frequent in samples with normal karyotype. Taking into account cases with AML-M2 and normal karyotype, the incidence of the V617F mutation was 10.8% (5/46). Two patients presented mutations in 2 different TK genes. Normal karyotype AML is a heterogeneous group with different molecular mutations, and a better subclassification of this group may be needed to work out a potential prognostic impact of TK mutations.

0634

PREFERENTIAL METHYLATION OF WNT INHIBITORY FACTOR-1 IN ACUTE PROMYELOCYTIC LEUKAEMIA: AN INDEPENDENT POOR PROGNOSTIC FACTOR

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Background. The Wnt pathway is activated frequently in acute leukaemia. *Aim.*to study the role of methylation of regulatory molecules in acute leukemia. *Methods.* The epigenetic suppression of Wif-1, a negative regulator of Wnt, was studied in five leukaemic cell lines and 107 acute leukaemia samples by methylation-specific PCR. *Results.* At diagnosis, Wif-1 was methylated in 25% (5/20) of acute lymphoblastic leukaemia. Interestingly, in acute myeloid leukaemia (AML) Wif-1 methylation was found only in acute promyelocytic leukaemia (APL) and not in other subtypes of AML (15/32, 47% versus 0/50, p=0.0001). In the APL cell line NB4 with hemizygously methylated Wif-1, treatment with the demethylating drug 5-azacytidine led to progressive increase in Wif-1 expression. In patients with APL, Wif-1 methylation was associated with younger median age (p=0.05) and higher presentation leucocyte count (p=0.03). The 3-year disease-free survival (DFS) of APL patients with Wif-1 methylation was significantly inferior to patients without Wif-1 methylation (36.0% versus 74.1%, p=0.005). In multivariate analysis, Wif-1 methylation. *Conclusion.* Wif-1 is preferentially methylated in APL, and may be an adverse prognostic factor.

0635

IMPLICATIONS OF REAL TIME FLIP GENE EXPRESSIONS ON THE CLINICAL OUTCOME IN ADULT PATIENTS WITH AML

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Background. Little is known about the expressions of Fas associated death domain-like interleukin 1α-converting enzyme-like inhibitory protein (FLIP) in normal hematopoietic in addition to leukemic cells. Many prognostic parameters have been developed to find a specific molecular explanation for unresponsiveness to induction chemotherapy (IC) in adult patients with acute myelogenous leukemia (AML). However, they should be on the considerations of pathogenetic heterogeneity of AML. *Aims.* Among several proteins in association with the process of anti-apoptosis on multiple levels by several regulatory mechanisms as a primary cause of treatment failure in AML, we focused on the levels of expression of FLIP. An attempt was made to correlate clinical outcomes with the expression of FLIP during IC because expression differences may be potentially important for predicting of response. *Methods.* A total of 76 bone marrow (BM) and peripheral blood (PB) samples from 32 fresh AML patients were collected after obtaining informed consent. Separately, 5 normal hematopoietic stem cell transplantation donors were used as a control. Acquisition of samples for analysis during IC and in the recovery period was routinely carried out at the initial diagnosis and on chemotherapy day 7, and after 21. Real-time PCR (RQ-PCR) was performed on an iCycler using iCycler software 2.1 (Bio-Rad, USA). RQ-PCR experiments were performed in duplicate, but if the FLIP-Actin values were discordant or inconsistent with RT-PCR result, the procedure was repeated. The initial response to IC together with each patient's FLIP expression levels was examined compared to the levels of normal population. Clinical profiles and results of RQ-PCR during treatment were compared. *Results.* Overall, AML patients, specifically at initial diagnosis, showed relatively higher FLIP levels of expression both in the BM and in the PB. BM showed relatively higher expressions than the PB in AML. Two significant values in association with clinical outcome were the levels of expression of FLIP at initial diagnosis and on day 7 BM/PB during IC. The most reliable one was at day 7 PB (p=0.02) and patients with complete remission (CRs) showed comparable expressions with those of normal control. An unexpected finding was that the expressions after immediate recovery had no correlations with outcome. The maximum expression difference of FLIP between CRs and poor responders was 1.2 log levels by RQ-PCR. *Conclusions.* Although further investigations with more patients are needed to verify the exact role of FLIP in a minor cell population as shown in this study, specifically at day 7 during IC, FLIP molecules may be an early prognostic clue for predicting CR in the treatment of AML patients, based on expressions of FLIP RQ-PCR.

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EXPRESSION OF THE BRAIN AND ACUTE LEUKEMIA CYTOPLASMIC GENE IN ACUTE MYELOID LEUKEMIA

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Clonal chromosome abnormalities play a major role in *de novo* AML being absolutely required to make a correct diagnosis and an accurate prognostic stratification. However, about 45% of AML patients are karyotipically normal and are supposed to have an intermediate prognosis even though only 40% of them are long-term survivors. Therefore, in these patients new molecular markers of prognostic significance have been actively searched and recent evidence suggests that BAALC gene expression levels are one of the most relevant. In the present study we determined BAALC expression in the pre-treatment bone marrow samples of 25 adult AMLs (7 M0-M1, 3 M2, 14 M4-M5 and 1 M6), 6 females and 19 males (median age 56 years, range 22-75). Eleven patients showed a normal karyotype, 2 a del(7)(q31q35), 2 a del(5)(q23q33) [one with +8], 2 a complex karyotype (33 abnormalities), 5 an inv(16)(p13q22) [two with +8], and 3 miscellaneous defects. The study was aimed at detecting the incidence of high BAALC expression and at correlating BAALC expression levels with clinical/biological parameters and outcome. Statistical analysis were carried out by applying the method of Pfaffl HW & Dempfle L (Nucleic Acids Res 2002;30:536). BAALC relative quantification was achieved with real-time PCR using SybrGreen I as a double-stranded DNA-binding fluorescent dye. The forward and reverse primers used were those already published (Baldus *et al.,* JCO 2006;24:790). Standard curve for real-time quantification was obtained by serial dilution of total RNA from an AML patient exhibiting an elevated BAALC expression. Quantification was achieved by applying the DDCt method. BAALC expression was normalized to ABL1 gene and calibrated on a normal control sample. A reference interval for BAALC expression quantification was fixed at 0,609 (mean expression [0,207] ± three times the standard deviation [0,134]) after having analysed 12 normal controls. BAALC expression was low in 11 patients (median ± 3SD = 0,158±0,561) and high in 14 (median ± 3SD = 5,427±11,691) with a statistically significant difference (p=0,001). No difference between the two groups was noted in pre-treatment age, sex, white blood cell count and percentage of bone marrow blasts. High BAALC expressers were predominantly of M4-M5 cytotype. Six of the 11 chromosomally normal patients were low expressers, whereas the 3 patients harbouring a single inv(16) and all the 3 with +8 were high expressers. Nine low expressers received induction

chemotherapy: 6 achieved a complete remission (CR) and 3 did not respond. Five of the 6 CRs relapsed and were unable to achieve a new CR. Eleven high expressers received induction chemotherapy: 8 achieved CR and 3 did not respond. Four of the 8 CRs relapsed but succeeded in achieving a second CR. In *Conclusion.* 56% of our AML patients presented a BAALC expression significantly higher than that of the remaining 11 patients; a high BAALC expression correlated with +8 and inv(16) (p13q22); high BAALC expressers showed a CR duration and an overall survival longer than those of low expressers perhaps because of the higher occurrence of +8 and inv(16) in the first patient group.

0637

THE OUTCOME OF POSTREMISSION TREATMENT FOR AML WITH FAVORABLE CYTOGENETICS IN FIRST REMISSION

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Background. The beneficial impact of high-dose cytarabine (HDAC)-based consolidation chemotherapy in acute myeloid leukemia (AML) is much greater in patients with favorable cytogenetics (t(8;21), inv(16) and t(16;16)) than in those with normal karyotypes. However, in MRC AML 10 study, patients with favorable cytogenetics who received autologous stem cell transplantation (SCT) had a markedly lower relapse rate than those who did not receive autologous SCT, although a high procedural mortality rate in adults resulted in being ultimately no difference in the overall survival (OS). Allogeneic SCT have not been recommended as standard therapy for AML with favorable cytogenetics due to relatively high treatment related mortality (TRM). However, progress in SCT and supportive care over the past decades have led to gradual improvement in the TRM after allogeneic SCT. *Aims.* We try to compare the outcome of allogeneic SCT with HDAC during the first remission of AML with favorable cytogenetics. *Methods.* 50 AML patients with favorable cytogenetics (excluded AML, M3) who entered complete remission (CR) between March 1997 and July 2005 at three centers were reviewed retrospectively. Among 50 patients, 13 patients who relapsed or died during consolidation chemotherapy, received less than three cycles of consolidation chemotherapy or underwent autologous SCT in first remission were excluded. Overall, 37 AML patients over the 18 years with favorable cytogenetics who underwent allogeneic SCT or received three/four cycles of HDAC consolidation chemotherapy in first CR could be analyzed. *Results.* The median follow up duration was 43 months. The 5-year probability of disease free survival (DFS) and OS were 50.3% and 51.6%, respectively. The estimated 5-year probability of DFS (73.2% vs 21.0%) (p=0.005) and OS (71.9% vs 28.9%) (p=0.03) were significantly better in the patients who underwent allogeneic SCT than in those who received HDAC. The cumulative incidence of TRM and relapse rate were 9.57% and 18.6%, respectively. In the subset analysis, OS was better in the allogeneic SCT group than in the HDAC group in the setting of age < 35 years (5-year estimated OS: 100% vs 33.3%) (p=0.0054), but not different in age ≥ 35 years (p=0.54). The OS was statistically superior for allogeneic SCT group versus HDAC group in the setting of chromosomal abnormalities ≥ 2 (5-year estimated OS: 72.9% vs 41.7%) (p=0.007), but not in chromosomal abnormalities < 2 (p=0.36). *Conclusions.* In AML patients with favorable cytogenetics (especially younger age) who have a matched related donor, allogeneic SCT can be option. Especially those who have more than 2 chromosomal abnormalities should undergo allogeneic SCT with matched related donor or unrelated donor. It is needed that AML patients with favorable cytogenetics who have sibling matched donor are assigned to allogeneic SCT and remaining to HDAC or autologous SCT are randomly assigned.

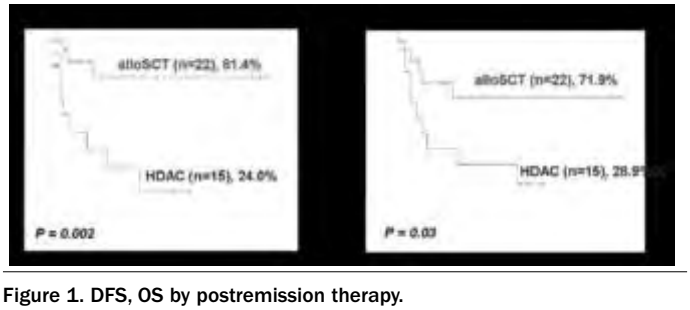

Figure 1. DFS, OS by postremission therapy.

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ARE FLT3 ITD AND D835 MUTATIONS SUFFICIENT INDICATORS FOR ALLOGENEIC TRANSPLANTATION IN ACUTE MYELOID LEUKEMIA? AN ANALYSIS OF PATIENTS FROM THE CZECH ACUTE LEUKEMIA CLINICAL REGISTER (ALERT)

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Background. FMS-like tyrosin kinase 3 (FLT3) is preferentially expressed on hematopoietic progenitor cells and mediates stem cell differentiation and proliferation. Two types of activating FLT3 mutations have been described in acute myeloid leukemia (AML): internal tandem duplication (ITD) of the FLT3 gene and point mutation within the activation loop of the tyrosin kinase domain, which mostly affects asparate 835 (D835). Many studies have shown that presence of FLT3 ITD correlates with poor outcome of AML patients. The prognostic relevance of D835 mutation is less clear, although most likely it also has a negative prognostic effect on the patients with AML. So far it is not clear how to treat the patients with FLT3 ITD and D835 mutations compare to patients without these mutations and whether these patients benefit from allogeneic blood stem cells transplantation. *Patients and Methods.* To assess the prognostic relevance of activating mutations of FLT3 gene on outcome of allogeneic transplantations in AML patients, we performed an analysis of all patients with FLT3 mutations registered in the Czech Acute Leukemia Clinical Register (ALERT) from 2003 till the end of 2005. ALERT registers all adult patients diagnosed in 6 main haematology centres in the Czech Republic. *Results.* Within the mentioned period 170 patients with AML of median age 59 years (in total) were investigated for FLT3 mutation, within them 37 cases (22%; 19 men and 18 women) with FLT3 mutations (33 FLT3 ITD and 4 FLT3 D835) were found. 33 patients were suitable for analysis. 13 of these patients had allogeneic transplantation, 20 patients with mutations of FLT3 were treated with chemotherapy without transplantation. Results of the treatment of these patients were compared with the results of the group of patients without FLT3 mutation, which was according to other characteristics identical with the group of patients with FLT3 mutations (n=125). *Results.* Median overall survival (OS) of patients with mutations of FLT3 who had allogeneic transplantation was 42.5 weeks, median survival of patients with mutations of FLT3 treated only with chemotherapy was 29.6 weeks (p=0.4). Median disease free survival (DFS) of the same patients was 32.1 weeks in transplanted patients and 24.3 weeks in patients treated only with chemotherapy (p=0.6). OS of patients with mutations of FLT3 compared to patients without mutation FLT3 was not significantly different. A significant difference was found in DFS only. Patients with FLT3 mutations had DFS shorter than patients without FLT3 mutations (28.2 weeks compare to 50.2 weeks; p=0.05). *Conclusions.* Our results suggest that at present there is no strong evidence that FLT3 status alone should influence the decision to proceed to allogeneic transplantation in AML patients. Decision to proceed to alogeneic transplantation should not be based on the FLT3 status only, but it should also consider other prognostic factors. Although the mutations FLT3 mean higher risk of relapses, according to our analysis they do not significantly influence the OS of AML patients.

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0639

OPTIMISATION OF A 48HOUR *IN VITRO* CHEMOSENSITIVITY ASSAY FOR CD34+CD38-CD123+ LEUKAEMIC STEM AND PROGENITOR CELLS

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Background. The majority of AML patients respond to remission-induction chemotherapy, but the relapse rate is high. Relapse is underpinned by outgrowth of leukaemic stem and progenitor cells (LSPC). There is a need to develop chemosensitivity assays for LSPC. *Aim.*We aimed to establish a methodology to distinguish normal from leukaemic SPC, to optimise maintenance of the LSPC phenotype in 48 hour culture and to quantify viable LSPC following culture with and without drugs. *Methods and Results.* The CD34+CD38-CD123high phenotype was used to

distinguish LSPC. CD123 fluorescence intensity was measured using fluorescence standards. LSPC were enriched from presentation samples using Miltenyi CD133-coated beads. The concentration of LSPC at the start of 48 hr culture ranged from 0.2-16×10^4/mL (median 3.4×10^4/mL). We compared various culture conditions aimed at maintaining these cells in culture without differentiation (i.e. without loss of phenotype), including serum, immobilized fibronectin, SCF, IL-3, IL-6, GM-CSF and angiopoietin 1. We used two flow cytometric assays in parallel for analysis of LSPC survival: in the first assay, viable bulk cells are enumerated using the dye 7-AAD along with an internal standard for cell counting. In the second assay, cells are labeled with CD34FITC, CD123PE, 7-AAD and CD38APC in order to quantify LSPC as a percentage of viable cells. We found that serum-free culture medium, fibronectin-coated wells, and a cocktail of SCF, IL-3, IL-6 and angiopoietin 1 was the most successful at maintaining the concentration of CD34+CD38-CD123+ cells in culture for 48 hours, (median 0% change), although there was considerable variation between samples. We examined the effect of these niche conditions on the sensitivity of LSPC to cytosine arabinoside (ara-C). Our preliminary data on 5 samples indicates that 500 ng/mL ara-C reduced the LSPC number to 15% of untreated control values in the absence of fibronectin and cytokines, whereas 50% of ara-C-treated LSPC were still viable in the presence of fibronectin and cytokines. *Conclusion.* We have defined a system for assessing the *in vitro* chemosensitivity of LSPC suitable for the study of anti-leukaemic agents in primary AML samples.

0640

EXPRESSION OF P73 AND P53 PROTEINS IN LEUKEMIC CELLS AND SURVIVAL OF ACUTE MYELOID LEUKEMIA PATIENTS

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Background. Prognostic significance of apoptosis-regulating proteins, especially recently discovered p73, is not clearly determined in acute myeloid leukemia (AML). The p73 protein is a new member of p53 family implicated in the regulation of cell cycle, apoptosis and development. Overexpression of p73 protein, with prevalence of short TAp73 isoforms, has recently been described in patients with AML. *Aims.* The main objective of this study was to verify whether expression of p73 and p53 proteins, pro- and anti-apoptotic members of the Bcl-2 family and caspase 3 has a prognostic impact on response to induction chemotherapy and overall survival (OS) of adult patients with AML. Additionally, we aimed to compare the expression of these apoptosis-regulating proteins between normal CD34+ and leukemic cells. *Material and Methods.* Intracellular expression of p73 protein in leukemic blasts isolated from bone marrow or peripheral blood was examined in 50 AML patients (36 *de novo*, 14 refractory/relapsed) of median age 55 years (range 28-78). In parallel, expression of other apoptosis-regulating proteins including p53, Bcl-2, Bax, as well as the cleaved form of caspase-3 as a marker of apoptosis, were studied. The control constituted CD34+ cells isolated from 10 Hodgkin lymphoma patients without bone marrow involvement. All measurements were performed using multi-color flow cytometry. Protein expression was expressed by both percentage of positive cells and mean fluorescence intensity. *Results.* Thirteen (36%) patients achieved complete response (CR) after induction chemotherapy, 20 (56%) patients did not respond and 3 (8%) patients died in the early post-induction period. The median time of the follow up reached 5 months (range 0.1-27). Comparing to normal CD34+ cells, AML blasts had higher expression of p73 and Bax proteins as well as cleaved caspase-3 (p<0.007, p<0.001 and p<0.0001, respectively), while no significant differences were noted regarding p53 and bcl-2. None of the analysed proteins showed predictive impact on probability of CR achievement after induction regimen. However, we found that AML patients with higher expression of p53 protein had significantly better OS as compared to other patients (14 vs 6 months, p=0.044). Moreover, similar trend towards longer OS was observed for the patients with higher expression of p73 protein (p=0.061). Interestingly, simultaneous high expression of both p53 and p73 proteins correlated with better overall OS of our AML cohort, as confirmed by univariate and multivariate analyses (p=0.012 and p=0.059; respectively). *Conclusions.* These data indicate that high expression of p73 protein in leukemic blasts, especially when co-expressed with p53 protein, favourably correlates with survival of adult AML patients. Furthermore, AML blasts may have increased proclivity to spontaneously undergo apoptosis comparing to normal CD34+ cells, with overexpression of proapoptotic p73 and Bax proteins.

0641

GENE EXPRESSION PROFILE OF ACUTE MYELOID LEUKEMIA WITH MULTILINEAGE DYSPLASIA CONFIRMS ITS BIOLOGICAL HETEROGENEITY

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Background. Acute myeloid leukemia with multilineage dysplasia (AML-MD), recognized in the WHO classification as a major AML category, is usually related to myelodysplasia and considered a poor-prognosis disease. However, the biology of this condition has not been extensively assessed, and previous studies suggest that cytogenetics defines different pathogenic and prognostic AML-MD subgroups. *Aim.* To analyze the gene expression profile of a series of AML with multilineage dysplasia (AML-MD). *Patients and Methods.* Nineteen patients (median age: 71, 30 - 93; 58% female) diagnosed with AML-MD in a single institution were included in the study. The gene expression profile of these cases at diagnosis was examined with oligonucleotide HGU133 Plus 2.0 arrays (Affymetrix). Expression measures were summarized using RMA methodology from the Affy package of the Bioconductor project. Unsupervised two-dimensional cluster analysis of high variability genes was done with dChip v1.3. In addition, a supervised analysis to identify genes with significant differential expression according to cytogenetic category was based on Limma package from Bioconductor which employs Bayesian statistics adjusted for multiple testing. *Results.* The unsupervised hierarchical cluster analysis identified two main groups of cases, which differentiated mainly according to cytogenetic risk category: group 1, (n=10), including 9 (90%) AML cases with intermediate-risk cytogenetics, and group 2 (n=9), with predominance of high-risk cytogenetics (78%, p=0.0045). Genes found overexpressed in group 1 included FLT-3 and several homeobox (HOXA3 HOXA5, HOXA7, HOXA9, HOXA10, HOXA11, HOXB2, HOXB3, HOXB5, HOXB6, HOXB7, HOXB8 and HOXB9) genes. On the contrary, relevant genes such as MLL, MLL3, CEBPD and EVI1 were overexpressed in group 2. The supervised analysis allowed the identification of a cluster of 92 genes differentially expressed according to cytogenetic category. Thus, genes found overexpressed in AML-MD with intermediate risk cytogenetics included ribosomal constituents and genes involved in translation (RPS20, LOC200916, LOC400055, EIF3S3), while diverse membrane-receptor genes, including genes involved in the immune response (FCGR3A, FCGR3B, IL1R2, PLXNC1, FCAR, CLEC4D, CLEC4E, TNFRSF10C, C5R1), were overexpressed in AML-MD associated with high-risk cytogenetics. The survival analysis of patients receiving intensive chemotherapy identified only cytogenetics, and not gene expression profile categories, as prognostically significant (figure). *Conclusions.* Gene expression profiling herein described supports the biological diversity of AML-MD, which seems to be related to the underlying cytogenetic abnormalities. Further studies in larger series of patients are warranted to gain insight into the biological diversity of this disease and clinical implications.

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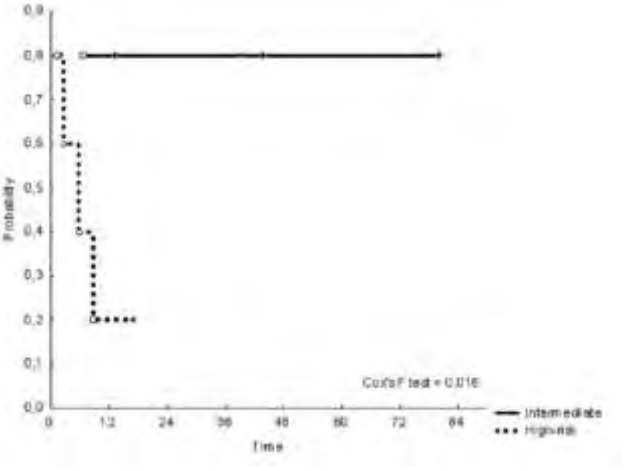

0642

THE USE OF BIOCHEMICAL MARKERS, ECHOCARDIOGRAPHY AND ELECTROCARDIOGRAPHY IN THE ASSESSMENT OF CARDIOTOXICITY IN PATIENTS TREATED FOR ACUTE LEUKEMIA

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Background. Cardiotoxicity is a relatively frequent and potentially serious complication of hematooncology treatment. Anthracyclines (ANT) represent the greatest risk. Cardiotoxicity of ANT may develop during the treatment (acute cardiotoxicity) and during the follow-up (chronic and late cardiotoxicity). Various methods including biochemical markers have been recommended for monitoring of cardiotoxicity of treatment in hematooncology. *Aims.* Monitoring of cardiotoxicity of ANT in patients treated for acute leukemia with biochemical markers ' N-terminal pro brain natriuretic peptide (NT-proBNP), cardiac troponin T (cTnT); echocardiography (ECHO) and electrocardiography (ECG). *Methods.* 26 adult acute leukemia patients (mean age 46.2±12.4 years, 15 males) treated with 2'6 cycles of ANT-based chemotherapy (CT) were studied. Cardiac evaluation was performed at the baseline (before CT), after first CT (cumulative ANT dose 136.3±28.3 mg/m^2), after last CT (cumulative ANT dose 464.3±117.5 mg/m^2) and circa 6 months after completion of CT (6 Mo after CT). *Results.* The results are summarized in the Table. Six months after CT, NT-proBNP concentrations correlated with systolic and diastolic LV dysfunction on ECHO ' (r=0,514; p<0.01) and (r=0,587; p<0.01). Decreased QRS voltage on ECG correlated with systolic and diastolic LV dysfunction on ECHO ' (r=0.660; p<0.001) and (r=0,592; p<0.01). *Conclusions.* Our results demonstrate acute and chronic cardiotoxicity of ANT. Clinical manifestation of cardiotoxicity in terms of heart failure developed in 2 (7.7%) patients. In asymptomatic patients, abnormal cardiac findings represent subclinical cardiotoxicity, which indicates a risk for development of heart failure (NT-proBNP elevations, diastolic LV dysfunction) and malignant ventricular arrhythmias (QTc prolongation). In regard of late ANT cardiotoxicity, further cardiology follow-up is warranted in all acute leukemia survivors.

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Table 1. Abnormal cardiac findings in patients treated for acute leukemia (n=26).

abnormal cardiac findings	*before CT*	*after first CT*	*after last CT*	*6 Mo after CT*
NT-proBNP elevation	3 (11.5%)	23 (88.5%)	23 (88.5%)	16 (61.55)
cTnT positivity	0	0	0	3 (11.5%)
systolic LV dysfunction	0	1 (3.8%)	1 (3.8%)	2 (7.7%)
diastolic LV dysfunction	1 (3.8%)	5 (19.2%)	6 (23.1%)	12 (46.2%)
QTc interval prolongation	1 (3.8%)	3 (11.5%)	7 (26.9%)	9 (34.6%)
QRS voltage lowering	-	31 (11.5%)	5 (19.2%)	6 (23.1%)

NT-proBNP elevation - NT-proBNP 100 pg/mL for male, 150 pg/mL for female; cTnT positivity - cTnT above 0,01 ng/mL; systolic LV dysfunction - EF bellow 55%; diastolic LV dysfunction - E/A inversion, DT above 220 ms; QTc interval prolongation - QTc above 440 ms; QRS voltage lowering - decrease in QRS voltage more than 1,0 mV.

0643

THE GROWTH AND SURVIVAL OF AML CELLS WITH T(8;21) ARE DEPENDENT ON VEGF/VEGF RECEPTOR TYPE2 AND PHOSPHORYLATION OF AKT

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Background. We have recently shown that AML cells having t(8;21) chromosome abnormality had augmented expression of vascular endothelial growth factor (VEGF) and type 2 receptor of VEGF (VEGFR2) (Leuk Lymphoma 47: 89-95, 2006). *Aims.* In this study, we examined the biological significance of VEGF/VEGFR2 system in AML cells. *Methods.* Two AML cell lines, Kasumi-1, having t(8;21) chromosome abnormality and NB4, having t(15;17) chromosome abnormality were studied. AML patient samples were also studied after the informed consent. Akt phosphorylation was determined by western blotting. The effects of VEGF165 and/or VEGF receptor2 kinase inhibitor were examined by MTS assay, cell count or annexin/PI assay. *Results.* First, we examined the phosphorylation of Akt, which is thought to be activated by VEGF, by different concentration of fetal calf serum (FCS). Kasumi-1 showed distinct Akt phosphorylation in a dose-dependent manner, although NB4 had undetectable level of Akt phosphorylation. Then, the Akt phosphorylation was almost completely inhibited by VEGF receptor2 kinase inhibitor, suggesting that Akt phosphorylation of Kasumi-1 by FCS was due to VEGF in FCS. Next, we checked the phosphorylation of Akt by the addition of VEGF165 in the culture of low FCS concentration (3%). Akt phosphorylation was augmented by the addition of VEGF165 in Kasumi-1, which was comparable to the phosphorylation status seen in Kasumi-1 cultured in 10% FCS. Finally, we examined the effect of VEGF165 and/or VEGF receptor2 kinase inhibitor on the growth of the cell lines. The addition of VEGF slightly augmented the growth of Kasumi-1. The addition of VEGFR2 kinase inhibitor greatly suppressed the growth of Kasumi-1 through induction of apoptosis although simultaneous addition of VEGF165 rescued the suppressive effect. On the other hand, the addition of VEGF165 with or without VEGFR2 kinase inhibitor did not show any significant influence on the growth of NB4. Then, patient samples were cultured with or without VEGF receptor2 kinase inhibitor. In accordance with the cell line study, all five AML cells with t(8;21) showed marked reduction of viable cells in proliferation (MTS assay) and increase of apoptosis cells by the addition of VEGF receptor2 kinase inhibitor. On the other hand, all four examined samples with t(15;17) did not show significant reduction of viable cells in proliferation by VEGF receptor2 kinase inhibitor. AML cells without chromosome abnormality showed various responses to VEGF receptor2 kinase inhibitor. *Summary.* These data strongly suggested that the growth and survival of AML cells with t(8;21) were dependent on VEGF through type 2 receptor of VEGF on leukemia cells, resulted in activation of PI3 kinase /Akt pathway. It is also demonstrated that the growth of AML cells with t(15;17) is not considered to be dependent on VEGF/VEGFR / PI3 kinase /Akt pathway. This kind of approach will be necessary to evaluate the effects of VEGF on leukemia patients for the VEGF-targeted therapy.

0644

A NOVEL MUTATION IN THE EXON 11 OF NUCLEOPHOSMIN (NPM1) GENE LEADS TO A TRUNCATED FORM OF THE PROTEIN LACKING THE C-TERMINAL NES-MOTIF

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Background. Aberrant cytoplasmatic expression of nucleophosmin (NPM1) due to mutations occurring at exon 12 of NPM gene has been associated to acute myeloid leukemia (AML). These mutations are the most frequent genetic abnormalities found in *de novo* AML with normal karyotype. They are often associated with Internal Tandem Duplication (ITD) of the FLT3 gene. All predicted mutant proteins carry at least one tryptophan amino acid at position 288 or 290. All but one showed the terminal amino acidic sequence VSLRK. Falini *et al.* recently demonstrated that all NPM1 mutant proteins carry a short stretch of hydrofobic amino acids, the nuclear export signal (NES) motif. The acquisition of the NES motif at the C-terminal and the loss of the tryptophan residue 290, are considered relevant to the cytoplasmic localization of NPM1 aberrant protein. *Aims.* DHPLC-based screening and sequence analysis of mutant products of the exons 9 to 12 of NPM1 gene in 102 AML patients, excluding M3 subtype. *Methods.* Bone marrow or peripheral blood samples were collected at diagnosis. RT-PCR for BCR-ABL, PML-RARalfa, AML1-ETO and CBFB-MYH11 were done. cDNA was used for the analysis of NPM1. Exon 12 of the NPM1 gene was screened using the NPM1_870F and NPM1_1112R primers. DHPLC analysis was conducted at 55.3 °C. Exons 9 to 11 were subsequently screened using the couple of primers NPM1_658F and NPM1_1112 R. DHPLC runs were performed at 54 °C and 55 °C. Only the homoduplex samples at the first round of DHPLC were tested. *Results.* All 26 mutant samples were negative for the searched translocations. Twenty-six (25.5%) amplicons were sequenced since they showed an heteroduplex profile. Type A (960_963dupTCTG) was the commonest observed change, occurring in 21/26 samples, followed by Type B (960_963insCATG) in 2 cases and Type IΔ (960_963insCAGA) in 1 case. We identified 2 novel sequence variants (VI1 and VI2), one of which in exon 11 of the gene (Table 1). Variant VI1 exhibited a 4 nucleotides insertion at position 958, leading to the acquisition of the most frequent NES motif type (LxxxVxxVxL). The mutation type VI2 showed an insertion of 8 nucleotides at position 902, in the middle part of exon 11. Nucleotidic insertion led to the creation of a stop codon at the level of the amino acid number 275 (Met274Stop). So, the truncated protein consisted of 274 amino acidic residues instead of 294 of the wild type. *Conclusions.* Variant VI2 is the only mutation described to date mapping outside the NPM1 exon 12. The predicted aberrant protein lacks the NPM C-terminal NES motif and do not contain neither tryptophan 288 nor 290. Further investiga-

tions would be important to understand the consequence of the lacking of the C-terminal NES motif in the nucleolar-cytoplasmic shuttle properties of the mutant protein VI2.

Table 1. Novel NPM1 mutations found in this study

Mutant type	Exon	Sequence	Predicted protein
WT	12	956-tc----tggcag----tggaggaagtctctttaa	286-DLWQWRKSL*
VI1	12	956-tctgttg----gcagtggaggaagtctctttaaggaaa	286-DLCLAVEEVSLRK
WT	11	892-gccaaattca--------tcaattatgtgaagaat	266-AKFINYVKN
VI2	11	892-gccaaattcaggcgcctatcaattatgtga	266-AKFRRLSIM*

GenBank Accession no. NM_002520; *, Stop codon

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EXTRAMEDULLARY INFILTRATES OF AML: BIOLOGICAL AND CLINICAL FEATURES IN A SINGLE CENTRE EXPERIENCE

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Background. Acute myeloid leukemia (AML) is a heterogeneous group of disease and AML patients may have distinct morphologic, cytochemical, immunophenotypic and clinical characteristics. Extramedullary infiltration (EMI) of malignant myeloid precursor cells may occasionally be a presenting clinical symptom at onset and may develop at any site in the body but most commonly in the gum, skin, central nervous system (CNS) and soft tissue. There is controversy about the prognostic significance of extramedullary disease in AML; while in some reports it confers a poorer prognosis other studies do not report a prognostic significance. Aim. The present study examines the incidence, biological features and prognostic significance of EMI at diagnosis in adult patients with AML. *Methods.* From January 1997 to December 2004, 213 untreated patients with *de novo* AML were studied; patients with APL were excluded. *Results.* Of 213 cases with *de novo* AML, 29 (13.6%) had EMI at diagnosis. Ten patients (34.8%) had skin infiltrates, 12 (41.4%) had gum hypertrophy, 5 (17.2%) had CNS involvement and 2 (6.9%) had soft tissue infiltration. No significant differences in terms of sex, age median Hb level and platelets count were found between patients with EMI and patients without EMI. The patients with EMI had higher median WBC counts (27 x 109/L) than patients without EMI (8.5 x 109/L) (p=0.05). The patients with EMI had a higher incidence of the M4/M5 FAB subtype (62%) than patients without EMI (27.4%) (p=0.005). Cytogenetic analysis was performed in patients with and without EMI; none of the abnormal cytogenetic findings was associated with EMI. We evaluated the relationship between the AML blasts surface antigen expression and EMI; the association between CD56/CD4 and CD56/CD14 was more significantly expressed in patients with EMI (35% and 29%, respectively) than without EMI (10.4% and 6.9%, respectively) (p=0.004, p=0.003). All patients had been treated with induction therapy according to the GIMEMA Protocols including Ara-C, etoposide and idarubicin (15 pts), mitoxantrone (15 pts) or daunorubicin (183 pts). The overall CR rate was 65%; the CR rate was lower in patients with EMI (48.2%) than those without (76.1%) (p=0.001) and their disease free survival was also shorter (p=0.017); the median duration of CR was 10 and 25 months (range 1-96) in the EMI and no EMI group, respectively. *Conclusions.* Our data demonstrate that a high WBC count, M4/M5 subtype, CD56/CD4 and CD56/CD14 expression are associated with extramedullary infiltrates of AML at diagnosis; the presence of EMI adversely affects the complete response rate to induction chemotherapy and the OS rate. Analysis of the clinical and biologic features in a larger series of adult AML patients is needed to evaluate the allocation of this subgroup to a different or more intensive treatment arm. Patients with EMI may warrant alternative therapy to improve their clinical outcome.

0646

SINGLE NUCLEOTIDE POLYMORPHISMS ANALYSIS OF TRANSCRIPTION FACTOR GENES IN ACUTE MYELOID LEUKEMIA

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Background. Acute myeloid leukemia (AML) has been proposed to arise from the collaboration of various chromosomal abnormalities. These chromosomal abnormalities found in AML frequently target the transcription factor genes which can control important biological processes, including cellular proliferation, differentiation, transformation and apoptosis. *Aims.* We selected 15 SNPs (single nucleotide polymorphisms) of transcription factor genes to test whether they are associated with increased susceptibility in patients with *de novo* AML. *Methods.* This study analyzed the frequencies of 15 SNPs of transcription factor genes in 269 *de novo* AML patients and age- and sex-matched controls. These 15 SNPs were selected from 339 SNPs analysis in previous study which were confirmed to be more than 15-20% in minor allele frequency in 120 normal Korean population. Genotyping method is pyrosequencing using genomic DNA from peripheral blood or bone marrow samples. *Results.* ETS2 rs530 (T1874A, OR: 1.929, range; 1.391~2.663), rs711 (G1+1655A, OR: 2.208, 1.596~3.504) and ELF1 rs7799 (A173G, OR: 1.949, 1.325~2.867) were found to be significantly higher frequencies of mutant genotype and allele in AML patients than in control. On the other hand, ELF1 rs1056820 (A1027T), ZNF42 rs4756 (A331G) and FLI1 grchd03-024 (C1-1014A) showed higher frequency of mutant genotype in AML than in control but there was no significant difference in mutant allele frequencies. *Conclusions.* This study showed the association between specific ETS family genes such as ETS2 and ELF1 transcription factors and AML prevalence. Therefore, it suggests these specific ETS gene abnormalities as a susceptibility gene in AML, and proposes a number of molecular strategies for targeting these genetic abnormalities for therapeutic intervention.

0647

CHARACTERIZATION OF AML-M0: A SEARCH FOR TUMOR SUPPRESSOR GENES AND ONCOGENES

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Acute Myeloid Leukemias (AML) form a heterogeneous group of hematologic malignancies partly characterized by specific translocations. Several oncogenes and only a few tumor suppressor genes (TSG) have been associated with AML. The search for TSG in leukemias has been to a certain extent neglected. In this study, we aim to better characterize the minimally differentiated AML subgroup (AML-M0). AML-M0 do not present specific cytogenetic abnormalities and generally have a poor prognosis. We studied cryopreserved cells from 52 AML-M0 patients. From this material we expanded T-cells to be used as control cells and sorted the leukemic blasts to obtain a pure tumor cell fraction. To find new TSG, we have used Affymetrix 10K SNP-microarrays to compare the DNA of the blasts with that of the control material. We searched for regions of loss of heterozygosity (LOH), as LOH is frequently the second *hit* resulting in the total loss of function of a TSG. Furthermore, we have screened the patients for mutations in known oncogenes such as FLT3, KIT, NPM1, NRAS, KRAS, PTPN11 and JAK2 and genes with dominant negative effect such as CEBPA .We found 16 patients with LOH of chromosome 21. Chromosome 21 harbors RUNX1, a well-known TSG frequently mutated in AML. We sequenced exons 3, 4 and 5 containing the Runt domain of RUNX1. 13 out of the 16 patients (81%) with LOH presented either a point mutation or deletion of RUNX1. Two other patients showed heterozygous mutations and another bi-allelic insertions. Thus in total 16 out of 52 patients (31%) showed mutation of RUNX1. We found 9 internal tandem duplications and 1 D835 activation loop mutation in FLT3 (19% of patients). From these patients only 2 cases had a RUNX1 mutation. From the 52 patients only one showed an insertion in exon 12 of NPM1. All other oncogenes mentioned above are currently being screened. Areas in chromosomes 3, 4, 7, 8 and 17 show LOH of DNA regions of less than 2 Mb and candidates TSG in these regions are currently under investigation. We hope that the characterization of AML-M0 will provide a better understanding and eventually a more favorable treatment of this disease, and will give insight into the pathogenesis of other subgroups of AML.

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STEM CELL TRANSPLANTATION IMPROVES OUTCOME IN YOUNGER ACUTE MYELOID LEUKEMIA PATIENTS WITH DETECTABLE MINIMAL RESIDUAL DISEASE AT THE END OF POST-REMISSION CHEMOTHERAPY

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Background. With modern chemotherapy, a complete remission (CR) is achieved in up to 90% of younger (<60 years) adult myeloid leukemia patients, but the majority of patients relapse due to persistence of minimal residual disease (MRD). Treatment strategies based on MRD status have not been established. *Aim.* The aim of the study was to evaluate the prognostic significance of multi-parameter flow cytometry (FCM) based MRD monitoring in relation to stem cell transplantation (SCT) in younger adult AML patients *Methods.* Between July 1994 and June 2001, 62 younger adult patients (<60 years) were diagnosed with non-promyelocytic AML at Karolinska University Hospital Solna (in Stockholm). Morphological CR was achieved in 53 of 62 patients (85%). Follow-up MRD information was available in 45 CR patients (23 males and 22 females). The diagnostic flow cytometry panel included membrane CD2, CD3, CD4, CD5, CD7, CD10, CD11b, CD13, CD14, CD15, CD19, CD20, CD33, CD34, CD45, CD56, CD65, CD117, HLA-DR, and intracellular CD3, MPO and TdT. Phenotypic aberrancies were defined at diagnosis and used in follow-up samples in three color antibody combinations as custom-built probes. MRD levels were determined after induction treatment (1) and after the end of post-remission chemotherapy treatment or before SCT (2). Detectable MRD was defined as a distinct cluster of at least 15 dots. Sensitivity levels were determined as: a) 0.1% if 30 000 events were acquired, b) 0.05% if 30 000 events were acquired in cases with highly aberrant LAIP as co-expression of CD34 and CD7, CD14, CD56 or CD65 and CD34+/CD15+/HLA-DR-, c) >0.01% if live-gate approach was used. ICE induction therapy was used in most patients (n=42). Autologous (auto-SCT) was performed as consolidation in first CR in 15 patients and allogeneic-SCT (allo-SCT) in 16 patients.

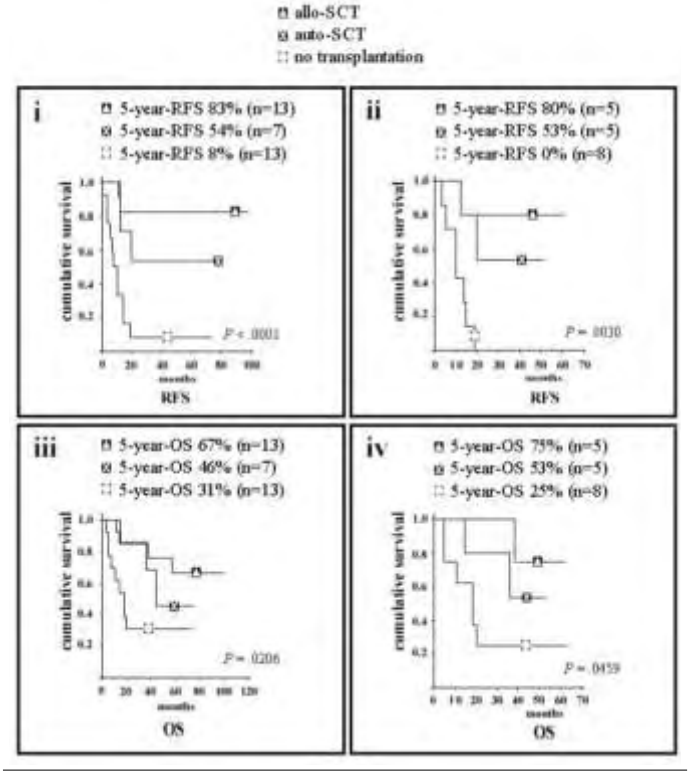

Figure 1. AML patients with detectable MRD after induction treatment (i,iii) or before SCT (ii,iv) subjected to allo-SCT had significantly longer RFS and OS as compared to patients who received auto-SCT or only conventional chemotherapy.

Results. Detectable MRD (1) did not predict relapse-free survival (RFS) and overall survival (OS) though there was a trend for longer RFS in MRD (2) negative patients (p=0.061). Improved RFS and OS were predicted only by SCT (p<0.001 and p= 0.001, respectively). To analyze in detail the impact of SCT on patient outcome, MRD positive patients were divided into 3 groups according to type of post-remission therapy: a) conventional chemotherapy, b) auto-SCT and c) allo-SCT. MRD (1) and/or (2) positive patients subjected to allo/auto-SCT had significantly better RFS and OS than patients who received only conventional chemotherapy (Figure 1). However, patients who underwent allo-SCT had a significantly better prognosis than patients who received auto-SCT. At time-point (2) 5-year RFS was 80%, 53% and 0% in allo-SCT, auto-SCT and no transplantation groups, respectively (p=0.003). *Conclusions.* Younger adult AML patients who have detectable MRD at the end of post-remission chemotherapy have a dismal prognosis and for these patients allo-SCT, auto-SCT or innovative new treatment strategies should be strongly considered.

0649

K313 DUP CEBPA MUTATION IS A RECURRENT MOLECULAR LESION IN *DE NOVO* ACUTE MYELOID LEUKEMIA

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The CEBPA gene code for a transcription factor with a pivotal role in the proliferation and differentiation control of the myeloid progenitors. Acquired CEBPA mutations are increasingly recognized in good prognosis acute myeloid leukemia (AML). Genomic DNA obtained from bone marrow samples of patients with MDS and AML referred to our hospital were analyzed by PCR-SSCP analysis. Samples with abnormal conformers were sequenced using both forward and reverse primers in an ABI310 genetic analyzer (Applied Systems, Foster City, CA). Forteen CEBPA mutations were detected in twelve patients. Interestingly, four cases showed an in-frame 3-bp insertion at the N-terminal region resulting in a duplication of the Lysine 313 (K313dup). All the four cases corresponded to *de novo* AML of the M1 FAB subtype. One patient carried also an out-of-frame C-terminal mutation consisting in a deletion of 10bp (T60fsX96). All the patients with mutations shared the following immunophenotype: CD15, CD34, HLA-DR, CD13, CD33 and CD7. CD7 reactivity has been previously associated with CEBPA mutations. All but one case had a normal karyotype. All the K313dup mutated patients were negative for AML1-ETO, CBFb-MYH11 and MLL rearrangements. One patient with the K313dup had also the D835 mutation of FLT3 associated with the following karyotype: 47,XY/46,XYdel(10)(q23q24),del(20)(q13). K313dup mutation has been reported and could represent as many as 10% of the total CEBPA mutations described to date. We used 3D-JIGSAW (Comparative Modelling Server) in order to obtain a 3D model of the wild type (wt) and the K313dup CEBPA proteins. RasTop 2.1 was employed to analyze the models. These systems predicted that the K313 duplication would change the a-helix structure of the protein. We were not able to identify Alu repeats both in the wt and the mutant CEBPA gene using the Repeat Masker Web Server (http://www.repeatmasker.org). Our results suggest that K313dup is a recurrent event in *de novo* AML. Sensitive assessment of K313 status in mutated cases would be a valuable tool in minimal residual disease studies. It remains to be investigated if this molecular lesion preferentially involves an immature myeloid precursor (CD34+CD7+CD33+).

Acute lymphoblastic leukemia

0650

SPECIFIC INTENSIVE CHEMOTHERAPY PLUS RITUXIMAB FOR BURKITT'S LYMPHOMA OR LEUKEMIA IN HIV-POSITIVE AND NEGATIVE ADULT PATIENTS

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Background. A previous PETHEMA protocol (PETHEMA ALL3/97) proved that HIV-positive patients with Burkitt's lymphoma (BL) and Burkitt-like acute lymphoblastic leukemia (ALL3) had similar outcome than HIV-negative patients. *Aims.* To study the impact of the addition of rituximab to our previous protocol in terms of toxicity and efficacy, with special attention to HIV-positive patients. *Patients and Methods.* All consecutive patients diagnosed with BL/ALL3 between July 2003 and January 2006 received induction therapy including a pre-phase with cyclophosphamide (CPM) and prednisone (PDN), followed by cycle A (rituximab, iphosphamide, VCR, dexamethasone -DXM-, HD-MTX, ARA-C and VM-26), cycle B (rituximab, VCR, HD-MTX, CPM, DXM and doxorubicin) an cycle C (rituximab, DXM, VDN, HD-MTX, HD-ARAC and VP-16). Patients with BL in stages I or II received 4 cycles (A1,B1,C1, A1) whereas those with BL in stages III or IV or with ALL3 received six cycles (A1,B1,C1,A2,B2,C2) followed by two additional rituximab doses. CNS prophylaxis consisted of IT MTX+ARA-C+DXM given in each cycle for a total of 8 doses. *Results.* 31 adult patients (20 HIV-negative and 11 HIV-positive) were included. Both groups of patients were comparable for age, gender, ECOG score, BM and CNS involvement, bulky disease, LDH and albumin serum levels. Twenty-two patients had BL and 9 ALL3. Three out of 11 HIV positive patients begun treatment with HAART at the time of diagnosis and 8 were already under treatment. Median follow-up was 7 months (range 1-30). Main results of therapy are summarized in Table 1.
No significant differences in CR, DFS or OS were observed between BL and ALL3 or between HIV-positive and negative patients. Grade 4 neutropenia and thrombocytopenia were constant and lasted a median of 7 days (range 2-31). Other frequent grade 3-4 toxicities were hepatic (8% of cycles), mucositis (18%) and infectious (18%). Episodes of grade 3-4 extrahematological toxicity were more frequent in HIV-positive patients (65% of mucositis, p=0.04; 65% of infections, p=0.04 and 62% of hepatic toxicities, p=NS).

	Total (%) n=31	HIV+ (%) n=11	HIV- (%) n=20
CR after two cycles	24 (77)*	7 (64)*	17 (85)*
Toxic death (1st two cycles)	4 (13)	2 (18)	2 (10)
Resistance	0	0	0
Toxic death in CR	2 (6)	2 (18)	0
Relapses during treatment	1 (3)	0	1 (5)
Relapses off treatment	0	0	0
Project. 1-year prob. DFS	87%	71%	93%
Project. 1-yr prob. OS	73%	57%	83%

Conclusions. Preliminary results suggest that the addition of rituximab to a specific BL/ALL3 treatment is also feasible for HIV-positive patients with similar results to HIV- negative patients in terms of efficacy although with higher toxicity.

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0651

MINIMAL RESIDUAL DISEASE ANALYSIS IN NON-MRD BASED TREATMENT PROTOCOL FOR CHILDHOOD ALL: LOW RISK FEATURES TOGETHER WITH FAST MORPHOLOGICAL RESPONSE FAIL TO IDENTIFY SLOW-RESPONDERS WITHIN THE ALL IC-BFM 2002 STANDARD RISK GROUP

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Since 2000, minimal residual disease (MRD) information at week 5 and 12 of therapy has been used for the treatment stratification in childhood ALL-BFM 2000 trial. In parallel, ALL IC-BFM 2002 has been designed by the International-BFM Group to test the morphological assessment of the early treatment response. Patients are stratified according to the blast proportion in peripheral blood (PB) at day 8 and in bone marrow (BM) at day 15 and 33 of therapy together with the age, initial WBC and the presence of BCR/ABL and MLL/AF4 fusion. *Aims.* One of the research questions of the ALL IC-BFM 2002 study is the comparison of this risk group assessment to the MRD-based criteria used in ALL-BFM 2000. *Methods.* MRD in BM or PB samples was assessed by patient-specific RQ-PCR for clonal immunoglobulin and T-cell receptor (Ig/TCR) gene rearrangements. *Results.* In total 184 patients treated according to ALL IC-BFM 2002 in the Czech Republic, Israel, Hong Kong and Uruguay were investigated for the presence of clonal Ig/TCR rearrangements. At least one patient-specific RQ-PCR target with minimal sensitivity of 10(-4) was designed for 161 patients. In these patients, MRD in BM at several time-points of therapy (including mandatory points at weeks 5 and 12) was evaluated; the PB specimens of Czech T-ALL patients were tested simultaneously. In total, 621 follow-up BM specimens and 80 PB samples were tested. The results showed separation of MRD levels between standard-risk group (SRG) and intermediate-risk group (IRG) stratified patients at day 33 (p=0.005). However, in 21 of 66 SRG patients (31.8%), MRD positivity at week 5 and/or at week 12 was observed (ranging from the positivity below QR to 1.5x10(-2)), thus identifying patients who would not qualify to the MRD-based SRG in ALL-BFM 2000 despite the identical induction regimen. Conversely, 24% of IRG patients showed MRD negativity by two independent Ig/TCR targets at both critical time-points, thus accomplishing the ALL-BFM 2000 SRG criterion. As expected, high-risk group (HRG) patients showed significantly slower molecular response than other groups. Taken together, patients with BCP ALL had significantly lower MRD levels at day 15 (p=0.03) and at day 33 (p=0.0004) than T-ALL patients. There was no significant difference in MRD levels between the two groups at week 12. In 80 follow-up T-ALL samples, MRD levels in PB clearly paralleled those in BM. *Conclusions.* Our findings revealed a significant difference between the stratification results of ALL IC-BFM 2002 and ALL-BFM 2000. Fast morphological response to treatment (M1 or M2 bone marrow at day 15) together with other low-risk features does not necessarily correspond with rapid MRD clearance.

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0652

MULTIVARIATE ANALYSIS INCLUDING MTHFR GENOTYPES IN A COHORT OF ALL PATIENTS

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Background. Several prognostic factors have been used to stratify ALL patients' risk. These prognostic factors include clinical and biological characteristics (age, WBC count, cytogenetic or molecular aberration and more recently the kind of early response to treatment). Recently the influence of polymorphisms of different genes involved in metabolism of chemoterapic agents have been studied expecially in childhood ALL. Methylenetetrahydrofolate reductase (MTHFR) catalyzes conversion of 5,10-methyltetrahydrofolate to 5-methyltetrahydrofolate in the folic

acid cycle. The C677T and A1298C MTHFR polymorphisms affect MTHFR enzyme causing a reduction of its activity, altered distribution of intracellular folate metabolites. Since MTHFR plays this important role in folate metabolism, differences in its activity due to these two gene variants might modulate therapeutic response to antifolate chemoterapeutic agents. In this study we evaluated the influence of classical prognostic factors and of C677T and A1298C MTHFR polymorphisms on time to relapse and survival in a group of 82 ALL patients. *Patients.* Patients' characteristics were: 46/36 M/F, median age 37.35 (12-75), B-phenotype L1-2 64pts, L3 7pts and T phenotype 11, normal karyotype in 45pts and high risk karyotype in 28pts. Forty-seven patients showed WBC>10×10^9/L at diagnosis. Forty-four pts relapsed at a median follow-up of 12 months (range 1-159) and 34 pts are alive at a median follow-up of 21 months (range 1-190). *Results.* The genotypes frequencies were consistent with previous published reports. The polymorphisms' distribution among different karyotype groups was homogeneous. On univariate analysis, pts with the MTHFR C677T and A1298C variant alleles did not experience significantly increased relapse and mortality risk (chi-square test p=0.82 and p=0.59 for 677 and p=0.36 and p=0.72 for 1298). Comparison of RFS and EFS between homozygous wild type and variant patients in both 677 and 1298 polymorphisms was not significant by the log rank test (p=0.79, p=0.53 and p=0.3, p=0.57 respectively), whilst RFS and EFS were significantly decreased in the presence of high risk karyotype and age >24y (p<0.0001 and p=0.03 respectively). The Cox regression analysis containing gender, age, WBC, karyotype, phenotype and MTHFR genotypes showed an increased hazard ratio (HR) relapse and mortality in patients with high risk karyotype (p<0.001 and HR 4.33 and p=<0.0001 and HR 3.67 respectively); an increased HR mortality was demonstrated in pts older than 24 years (p<0.001 and HR 0.415). Regarding WBC count at diagnosis there was no significant correlation between WBC>10x109/L and outcome whilst we found an increased risk of mortality among patients with WBC>50×10^9/L (chi-square test p=0.006). *Conclusions.* In our study we did not observe any association between MTHFR polymorphisms and relapse and survival rate in a group of almost adult ALL patients. Our data are in contrast with those from other groups which evaluted the influence of these two polymorphisms in pediatric standard risk patients. Due to the higher frequency of molecular alterations (t9;22 and t4;11) in our contest MTHFR polymorphisms per se has not enough power to influence DFS and EFS, when compared to classical risk factors like karyotype aberrations, WBC at diagnosis and age influencing prognosis.

0653

DASATINIB IN PATIENTS WITH CHRONIC MYELOGENOUS LEUKEMIA IN LYMPHOID BLAST CRISIS OR PHILADELPHIA CHROMOSOME POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA THAT IS IMATINIB -RESISTANT OR INTOLERANT : THE CA180015 START-L STUDY

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Background. Dasatinib (D) (BMS-354825) is a multikinase inhibitor of Bcr-Abl and SRC. In a phase I study, hematologic responses were achieved with D in pts with LB-CML and Ph+ALL. *Aims.* To estimate the major hematologic response (MaHR) rates to D in IM-R and IM-I patients with LB-CML and Ph+ALL. *Methods.* START L is an open label phase II study of D in IM-R or IM-I pts with LB-CML and Ph+ALL which was conducted in 42 centers worldwide. D was given orally, 70 mg twice a day (bid), with escalation to 100 mg bid for inadequate initial response or reduction to 50 mg and 40 mg bid for toxicity. Pts had weekly blood counts and monthly bone marrow (BM) exams, including cytogenetics. Molecular evaluation of Bcr-Abl transcripts was performed by real-time quantitative PCR at baseline and at complete cytogenetic response. Bcr-Abl mutations were assessed at baseline and at time of progression. The primary endpoint was confirmed (sustained for at least 4 weeks) major hematologic response rates (MaHR). *Results.* From January to June 2005, 94 pts were treated. Data are available on the first 42 LB-CML and 36 Ph+ ALL treated pts. Of the 48 LB-CML pts, 42 had IM-R, 52% were male with median age of 49 yrs (range 17'73). Prior therapy included IM >600 mg/day in 52% pts and stem-cell transplant (SCT) in 31% pts. Median baseline platelet (plt) count was 35/nl, median BM blasts was 82%. Of the 46 Ph+ALL pts, 44 were IM-R, 59% were male with median age of 47 yrs (range 15'85). Prior therapy included IM> 600 mg/day in 45% pts and SCT in 37% pts. Baseline plt count was 62/nl, median BM blasts were 70%. Preliminary efficacy and safety analyses are currently available on the first 78 pts. Among the 42 LB-CML pts, the D dose was reduced in 14%, temporarily interrupted in 33%, and escalated in 26% of pts. At 6 months, the MaHR rate was 31% including 26% complete hematologic response (CHR), the MCyR rate was 50%, and 17% pts remained on study. Among the 36 Ph+ALL pts the D dose was reduced in 28%, temporarily interrupted in 39%, and escalated in 47% of the pts. At 6 months, the MaHR rate was 42% including 31% CHR, the MCyR rate was 58%, and 33% pts remained on study. Among all 78 pts, grade 3-4 thrombocytopenia and neutropenia were seen in 82% and 76% of pts, respectively. The most frequent D-related non-hematologic toxicities were diarrhea (30%), nausea (23%), fatigue (19%), rash (17%) and pleural effusion (13%). *Conclusion.* D has substantial activity in heavily pretreated LB-CML and Ph+ALL pts. Data on all 94 pts will be presented at the meeting including an analysis of the molecular response and Bcr-Abl mutations.

0654

CRYPTIC KARYOTYPE DEFECTS ARE DISCOVERED BY INTERPHASE FLUORESCENCE *IN SITU* HYBRIDIZATION (I-FISH) IN CHROMOSOMALLY NORMAL ADULT B-CELL ACUTE LYMPHOBLASTIC LEUKEMIA (B-ALL).

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In adult B-ALL the chromosome pattern plays a pivotal role in the prognostic stratification of patients and in driving therapeutic decisions. Unfortunately, conventional cytogenetics (CC) is not always informative since it is often hampered by either the absence of mitotic cells or by the bad quality of metaphases. Thus, FISH, which can be performed on mitotic as well as on quiescent cells, has progressively been used in addition to CC to unmask chromosomal changes and cryptic defects in B-ALL. We have applied I-FISH to analyse 38 adult B-ALLs who showed a normal chromosome pattern on G- and Q-banded metaphases. Our study was aimed at establishing the true incidence of BCR-ABL, ETV6-AML1, MLL rearrangements and p16/INK4A deletion in chromosomally normal B-ALL and at correlating our findings with clinical parameters and outcomes. The 38 patients examined, 22 females and 16 males with a median age of 41 years (range 16-78), were part of a large series of 263 consecutive adult B-ALLs who came to our observation in a ten years period (1994-2005). Within this series 56 patients (21,2%) did not yield metaphases and 73 (27,7%) presented a normal chromosome pattern. I-FISH was carried out with the following commercial probes: LSI BCR/ABL1 dual color single fusion, LSI TEL/AML1 ES, LSI MLL and LSI p16 (9p21)/CEP 9 dual color, which is 190kb long and spans a number of genetic loci including D9S1749, D9S1747, p16(INK4A), p14(ARF), D9S1748, p15(INK4B) and D9S1752 (Vysis, Downers Grove, IL, USA). Hybridization procedures were carried out according to manufacturers' guidelines. Cut-off values were determined after having analysed two-hundred cells from ten normal controls and using a one-sided binomial distribution with a 95% confidence interval. So, the cut-off values were fixed at 10% and 6% for the BCR/ABL1 and MLL probes and at 3% for both the ETV6-AML1 and the LSI p16 (9p21)/CEP 9 probes. I-FISH discovered clonal chromosome defects in a total of 17/38 (44.7%) patients. The loss of either one or two red spots corresponding to the LSI p16 (9p21)/CEP 9 dual color probe was the most common cryptic abnormality, being observed in 10 patients (26,3%). No patient presented a cryptic BCR-ABL or ETV6-AML1 rearrangement. The amplification of the AML-1 gene and the monosomy of the ETV6 gene were observed in 11-15% cells from 3 and 2 patients, while the monosomy and the amplification of the MLL gene in one patient each. The 2 patients with p16 nullisomy were unresponsive to chemotherapy and survived four and six months; those with p16 monosomy were too few to obtain any prognostic information. *Conclusion.* i) I-FISH in combination with CC is a very useful tool to unmask cryptic defects in B-ALL since it readily discovered genetic aberrations in 44% of our chromosomally normal patients, ii) p16/INK4a monosomy/nullisomy is a very common defect not only in T-ALL but also in B-ALL, iii) ETV6-AML1 and MLL rearrangements are extremely rare in adult B-ALL, iii) CC is very effective in detecting Ph positive cells although the clonal cell population is more accurately defined by I-FISH.

0655

WILMS TUMOR GENE 1 EXPRESSION IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

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Background. Wilms' tumor gene 1 (WT1), located on chromosome 11p13, encodes a zinc-finger transcription factor with important roles in embryogenesis and oncogenesis. WT1 is supposed to be overexpressed in the majority (70-90%) of acute leukemias and has been identified as an independent adverse prognostic factor, a convenient minimal residual disease (MRD) marker and potential therapeutic target in acute leukemia. However, among the increasing number of studies, results some of them regarding WT1 expression and its clinical impact become controversial. Many of the discrepancies could be attributed to the non-standardized techniques of WT1 detection and quantification, different patients' characteristics and limited number of samples and controls investigated. *Objectives.* The aim of this study was to establish reproducible PCR assays for WT1 detection and evaluate WT1 expression in a representative group of childhood ALL patients. *Methods.* RT-PCR and RQ-PCR enabling absolute quantification of total WT1 and its four main isoforms (variants A, B, C and D) were designed, optimized and validated according to BIOMED-1 Concerted Action [van Dongen *et al.,* Leukemia 1999] and Europe Against Cancer Program [Gabert *et al.,* Leukemia 2003] recommendations, respectively. With these methods we evaluated WT1 in diagnostic bone marrow (BM) samples of 125 consecutively enrolled childhood ALL patients (106 BCP-ALL, 19 T-ALL); normal peripheral blood (PB) and BM, and regenerating MRD negative BM were used as controls. *Results.* Low WT1 expression of a uniform pattern was present in all control samples. In BCP-ALL, we detected a wide range of WT1 levels (5 logs) with median close to that of normal BM; WT1 expression in T-ALL was significantly higher ($p<0.001$). Patients with MLL-AF4 translocation showed considerable WT1 overexpression ($p<0.01$) compared to other patients. Older children expressed higher WT1 levels than children under 10 years of age ($p<0.001$), while there was no difference between patients with WBC over 50×10^9 /L and lower. There was also no correlation between WT1 and CD34 expression. Analysis of relapsed cases (14/125) indicated that abnormal increase or decrease in WT1 expression was associated with significantly increased risk of relapse ($p=0.0006$), and this prognostic impact of WT1 was independent of other main risk factors ($p=0.0012$). All four WT1 isoforms were detected in normal controls and ALL samples. Preliminary results did not show a significant difference in WT1/exon5[+] and WT1/KTS[+] ratio in ALL patients compared to normal BM. *Conclusion.* In summary, WT1 expression in childhood ALL is variable and much lower than in AML or adult ALL. WT1 thus will not be a useful marker for MRD detection in childhood ALL, however, it does represent a potential independent risk factor in childhood ALL. Interestingly, a proportion of childhood ALL patients express WT1 at levels below the normal physiological bone marrow WT1 expression, and this reduced WT1 expression also appears to be associated with a higher risk of relapse. The designed RT-PCR and RQ-PCR assays for detection of WT1 and its main isoforms could be considered as standards for future reference and use.

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CELL BIOLOGICAL FEATURES OF BLASTS PERSISTING AT DAY 8 OF INDUCTION THERAPY IN CHILDHOOD PRECURSOR B-CELL ACUTE LYMPHOBLASTIC LEUKEMIA

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In childhood acute lymphoblastic leukemia (ALL), persistence of leukemic blasts during therapy is of crucial prognostic significance. In the frontline ALL-BFM (Berlin-Frankfurt-Münster) trial, treatment stratification is based on blast count estimation in peripheral blood at day 8 of induction prephase with prednisone and one intrathecal dose of methotrexate. Recently, we investigated genome-wide gene expression of blasts persisting after one week of induction therapy (day 8 blasts). The observed expression changes in day 8 blasts as compared with blasts at initial diagnosis (day 0 blasts) included key regulators of the cell cycle and genes encoding for B-cell differentiation markers. Furthermore, we observed an expression increase of inflammatory response genes and a decrease of BCL2, the prototypic member of the anti-apoptotic BCL2-subfamily. In the current study we analyzed day 8 blasts at protein and cellular levels. Firstly, we isolated the day 0 and day 8 blasts of 13 patients by flow sorting and measured the cell cycle distributions at both days. As a result, mean percentage of cycling (S-, G2/M-phases) cells in the blast subpopulations significantly decreased from 5.1% (range: 0.2-22%) at day 0 to 1.2% (range: 0.1-5.1%) at day 8 ($p=0.014$). In a total series of 56 patients, flow cytometric analysis confirmed expression changes of the B-cell differentiation markers CD10 (decrease by 1.4-fold), CD20 (increase by 2.4-fold), CD34 (decrease by 1.3-fold) and TdT (decrease by 2.4-fold) ($p<0.005$). Moreover, we were also able to confirm the expression increase of the inflammatory response molecules CD11b (5.1-fold, $p=0.001$, n=15) and IFNGR (2.2-fold, $p<0.001$, n=15), and the decrease of the BCL2 protein (1.5-fold, $p<0.001$, n=29). Taken together, the cell biological characterization of ALL cells persisting during induction therapy demonstrated an inhibited cell proliferation and an overall gene expression shift towards resting mature B cells. Furthermore, expression decrease of BCL2 in the day 8 blasts points to the involvement of this anti-apoptotic protein in the molecular mechanism of action of glucocorticoids in childhood ALL.

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MINIMAL RESIDUAL DISEASE MONITORING OF BCR-ABL POSITIVE ADULT ACUTE LYMPHOBLASTIC LEUKAEMIA USING QUANTITATIVE REAL-TIME PCR: PRELIMINARY RESULTS FROM THE MRC-UKALLXII TRIAL

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Background. Adult ALL carrying the BCR-ABL fusion-gene is associated with a dismal prognosis. Minimal residual disease (MRD) is a significant tool for monitoring disease progression and outcome and BCR-ABL offers a convenient target for molecular MRD monitoring by QRT-PCR. In 2003, the MRC UKALLXII trial was amended to incorporate the addition of the tyrosine-kinase inhibitor Imatinib Mesylate for BCR-ABL+ patients during intensification. 25-30% of adult ALL patients exhibit BCR-ABL and the p190BCR-ABL isoform is more common than the p210BCR-ABL. It remains to be seen whether isoform type carries clinical significance. *Aims.* To investigate the efficacy of monitoring response to induction therapy and Imatinib in adult ALL patients enrolled on the MRC-UKALLXII trial using BCR-ABL as a target for molecular MRD. *Methods.* MRD analysis was performed using Roche LightCycler 1.2 with SYBR-Green fluorescent technology and primers for ABL, p210BCR-ABL and p190BCR-ABL. BCR-ABL expression was normalised ratiometrically against ABL. Four time points for analysis were taken: presentation, post-induction (pre-Imatinib), post-intensification (post-Imatinib), and following BMT. *Results.* 395 samples (182 PB, 197 BM, 16 cDNA) were received from 55 BCR-ABL+ patients. 40 (75.6%) were positive for the p190BCR-ABL isoform and 16 (24.4%) for the p210BCR-ABL. There were 39 male and 16 female patients, median age of 44y (range 17-64y) with a median WBC of 23.3×10^9/L (0.5-428.0). There was no difference in patient age or sex between isoforms, but WBC at presentation was found to be significant: p210BCR-ABL = 52.7 (range 2.4-428.0) and p190BCR-ABL = 16.7 (0.5-301.0) ($p=0.0221$). 32 patients (58.2%) received Imatinib and 24 (43.7%) a BMT. Statistical analysis of median BCR-ABL expression showed a significant drop in BCR-ABL levels between each time point ($p<0.0001$, $p=0.0005$ and $p=0.0199$) and that p210BCR-ABL patients exhibited significantly higher levels of BCR-ABL than p190BCR-ABL patients at each time point ($p=0.0108$, $p=0.0123$ and $p=0.0196$). *Summary.* PB and BM samples were found to be equivalent in sensitivity, except for the pre-Imatinib samples, where BM was found to offer significantly higher sensitivity ($p=0.0252$). Although BCR-ABL levels decreased in response to induction therapy and in response to Imatinib, patients with the P210 isoform had consistently higher transcript levels than patients with the P190 isoform. MRD in adult ALL is shown to be a significant indicator of disease progression and as such may be used to implement treatment stratification.

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SHOULD HYPERCVAD/METHOTREXATE-CYTARABINE BE CONSIDERED A STANDARD TREATMENT IN ACUTE LYMPHOBLASTIC LEUKEMIA? A BRAZILIAN EXPERIENCE

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Background. Adult ALL is traditionally treated by a Vincristine and Prednisone protocol with the addition of a Anthraycline. Early dose intensification as described by the MD Anderson group with the hyperfractionated Cyclophosfamide, Vincristine, Doxorubicin, Dexamethasone/high dose methotrexate-cytarabine regimen has shown in there experience an improvement in disease free survival. There are few data about the experience with this high dose protocol from other groups. *Methods and Results.* We analysed retrospectively 65 patients treated between 1994 and 2005 in 3 brazilian hospitals. Median age was 21 years; only 3 patients were > 60 years. The incidence of philadelphia positive ALL was 6% and T-ALL 23%. According to age, leucocyte count, SNC disease at diagnosis, remission after first cycle, patients were classified as high risk (69,5%) or low risk (30,5%). Overall 54 (80%) patients achieved complete remission after the first chemotherapy cycle. The median time between treatment cycles was 56 days (range 27 - 83) mainly because of severe infections. Recurrence of the disease occurred in 5 patients (7%) during induction. Forty patients (61%) were able to complete the 8 induction courses. Twenty three (33%) patients died during induction and 2 patients changed chemotherapy protocol because of severe toxicities. Infection was the most common cause of death. From the 40 patients who finished the induction courses only 25 were able to receive maintenance. Of the remaining 15 patients, 10 relapsed before maintenance and 5 died because of infectious complication. At a median follow up of 12 months, only 22 (34%) of the patients are alive. Three more patients relapsed during maintenance. Main cause of death was infection and relapse of the disease. *Conclusions.* We were not able to reproduce the favourable data published for this protocol. The intensification during the induction phase with high dose cytarabine and methotrexate is in our experience very toxic and related with a high proportion of early deaths. The occurrence of severe infections and toxicities (especially neurological), who were responsible for the high rate of induction deaths (33%), also results in a delay in treatment and consequently in relapse of the disease. Several patients had to interrupt the protocol and start another treatment regimen. The better stratification of patients, the use of a less toxic induction regimen and subsequent risk adapted intensification, seems in our view a better approach for ALL patients.

0659

IMATINIB COMBINED TO INDUCTION OR CONSOLIDATION CHEMOTHERAPY IN YOUNGER PATIENTS WITH *DE NOVO* PHILADELPHIA CHROMOSOME-POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA RESULTS OF THE GRAAPH-2003 STUDY

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Combination of imatinib with standard chemotherapy has been recently reported as very promising in patients with Ph+ ALL. Between January 2004 and October 2005, 45 patients with newly-diagnosed Ph+ ALL (median age, 45 years; median follow-up, 11 months) were treated in the GRAAPH-2003 study in which imatinib was started with HAM consolidation in good early responders (cortico- and chemo-sensitive ALL) or earlier during the induction course in combination with dexamethasone and vincristine (DIV regimen) in poor early responders (cortico- and/or chemo-resistant ALL). In all patients, imatinib was then continuously administered until stem cell transplantation (SCT), either allogeneic if aged 55 years or less with a matched familial or unrelated donor, or autologous if older or no donor but in molecular remission. Overall, hematological and molecular remission rates were 96% and 62%, respectively. The rate of patients able to receive SCT as planned by the protocol was 65%. At 18 months, estimated disease-free and overall survival was 51% and 65%, respectively. All these endpoints compared very favorably with results obtained in the pre-imatinib LALA-94 trial. Interestingly, the bad prognosis previously associated with a poor early response to standard therapy, a lack of allogeneic donor, and a non-achievement of molecular remission was not evidenced in this study. In conclusion, this study confirms the value of the combined approach in younger patients with Ph+ ALL and encourages prospective trials to define the optimal chemotherapy which has to be combined with imatinib and carefully re-evaluate the place of allogeneic SCT in this new context.

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ROLE OF P21 IN ADULT ACUTE LYMPHOBLASTIC LEUKEMIA PATIENTS: METHYLATION STATUS AND PROTEIN EXPRESSION.

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Background. Methylation of CpG islands in the 5' gene region is associated with transcriptional silencing of gene expression. The hypermethylation of tumor suppressor genes has been described in gastric and pancreatic cancer, as well as in acute myeloid leukemia, suggesting its potential role in neoplasia. Among the three members of the Kip/Cip family of cyclin dependent kinase inhibitors (CKI) p21, p27 and p57, little is known about their methylation status in hematological malignancies. Contrasting studies, have been reported on the role of p21 hypermethylation in acute lymphoblastic leukemia (ALL). *Aims.* To analyze p21 gene methylation status and protein expression in primary blasts from adult ALL enrolled in the GIMEMA protocol LAL2000. *Methods.* Human leukemic cell lines, normal peripheral blood lymphocytes (PBL) and 89 primary samples from untreated ALL patients were evaluated in this study. The p21 gene methylation status was investigated using a widely accepted method based on bisulfite modification of DNA, followed by the use of methylation-specific PCR assay (MSP). This assay was further validated *in vitro* by SSI methylase. The p21 protein expression was analyzed by Western blot using the p21-WAF1 MoAb (Santa Cruz, CA). *Results.* The human lymphoblastic cell lines RPMI8866 and CEM, the myeloid cell line OCI-AML3 and normal PBL from 10 healthy donors were characterized by a consistent p21 promoter unmethylation (negative controls). In contrast, a weak methylation was documented in the Raji and Jurkat cell lines, while the Rael (Burkitt's lymphoma) cell line was strongly methylated (positive controls). In addition, p21 protein expression was found in the OCI-AML3, Raji and RPMI8866 cell lines, while it proved negative in the Jurkat and Rael cell lines, and in normal PBL. Sixty primary ALL cases evaluated for p21 methilation status showed a consistent unmethylation in all samples, while the p21 protein expression was found in 26/89 cases (29.2%). A significant correlation (p=0.010) was observed between p21 protein expression and immunophenotype, 37.5% of B lineage ALL compared to 8.3% of T lineage ALL. In addition, a trend was found between p21 expression and age. Achievement of CR was observed in 65.4% and 79.4% of p21 positive and negative cases, respectively. *Summary.* While p21 gene methylation does not appear to play a pathogenetic mechanism in adult ALL, p21 protein expression is found in one third of these patients suggesting a role in the disease.

0661

PROGNOSTIC VALUE OF HOX11L2/TLX3 AND TAL1/SCL EXPRESSION IN CHILDHOOD T-CELL ACUTE LYMPHOBLASTIC LEUKAEMIA : RESULTS OF THE FRALLE 93 PROTOCOL

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B*ackground and aim of the study.* The most frequent oncogenic activation events characterized in childhood T acute lymphoblastic leukemia (T-ALL) result in the transcriptional activation of genes coding for transcription factors. The main genes are TAL1/SCL, a member of the basic region helix-loop-helix gene family, HOX11L2/TLX3 a member of the homeobox-containing protein family. Conflicting results have been reported concerning molecular epidemiology and prognostic values of these markers. We therefore analysed retrospectively 200 patients treated in the French protocol FRALLE 93 for T-ALL between 11/93 and 12/99. *Methods.* Patients were stratified according to prednisone response at D8 (good or poor : GPR or PPR) and bone marrow at D21. Pts with D8 PPR or M3 received an intensified treatment with genoidentical or autologous transplant in CR1. Molecular analysis was done in 120/200 T-ALL samples. *Results.* Clinical caracteristics were not significantly different between population with or without molecular analysis: male (n=121) (69% vs 70%), median age 8.4y (range1.1-19.5) vs 9.2y, median leucocytosis 140.109 (0.6-736) vs 171.915 109 (<50 n=23, 50-99 n=8, >100 n=48), mediastinal involvement 72% vs 71% ,CNS+ 4% vs 5%, CD 10 neg 54% vs 52%. Steroid response PPR n=37/73, GPR n=36/73 and D21 bone marrow status (M3 n=10, M2 n=11) were similar. CR was obtained in 105/118 pts (90%) after first induction therapy and 2 deaths occurs during induction treatment. With a median follow-up of 63 months (2-123), 5 y OS , EFS and DFS is 62% 9 and 54% 10. SIL-TAL1/SCL fusion was detected in 16.6% (20/120) pts; expression of TLX3 was observed in 23% (20/87) pts. The incidence of HOX11/TLX1 activation , CALM-AF10 and NUP 214-ABL1 rearrangements were of 3.5% (3/83), 5.4% (4/74) and 7.7% (4/52) respectively. These events were mutually exclusive, except for NUP214-ABL1 which significantly associate with TLX3, and allow the classification of near 50% of the patients. We also searched for NOTCH1 point mutations in the subset of TLX3 positive leukaemia. Mutations were found in 80% of cases: half of them had known activating mutations the others had heterozygous or homozygous SNP in HDC or TAD domains. Median leucocytosis was significantly higher in SIL-TAL1/SCL pts (p=0.01) but other significant features (ie median age, D8 response, D21 status) were not significantly different between each group. OS and EFS for TAL1/SCL , HOX11L2+ and none of these were respectively 81% 10, 43% 13, 62% 7 and 80% 10 , 42% 13, 55% 7. OS and EFS D8 PPR/GPR were 47% 9 vs 78% 7 (p=0.009) and 41% 8 vs 71% 7 (p= 0.008); OS and EFS M2M3 vs M1 D21 bone marrow status were 45% 15 vs 70% 6 (p=0.05) and 36% 10 vs 66% 6 (p= 0.018). In multivariate Cox model analysis, D8 steroid response and TLX3 expression were significantly associated with adverse event (failure or relapse) Risk Ratio :3 and 2.6 - p=0.008 and 0.03 and a higher risk of death Risk Ratio : 4.1 and 3.4 -p=0.061 and 0.05. *Conclusion.* TLX3 expression and D8 PPR are independently associated with poor outcome in FRALLE 93 protocol analysis which confirms our previous report.

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QUANTIFICATION OF DRUG INDUCED CASPASE ACTIVATION IN PRIMARY LEUKEMIA CELLS *IN VITRO* AND *IN VIVO* BY FLOW CYTOMETRY

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Background. Defects in apoptosis signaling are involved in leukemogenesis and may be responsible for drug resistance and treatment failure. As activation of the effector caspase-3 represents a key component of apoptosis signal transduction caspases are most advantageous targets to detect and quantify apoptosis signaling. The lack of convenient methods for the measurement of activated apoptosis signaling in primary leukemia cells hampers the analysis of apoptosis pathways for drug sensitivity or resistance. *Aim.* Quantification of key apoptosis signaling events such as caspase activation for analysis of apoptosis and drug resistance in primary leukemia cells *in vitro* and *in vivo*. *Methods.* We developed and characterized a flow cytometric method for a quantifying measurement of caspase activation by cleavage of derivatives of (Asp)2-rhodamine 110 (D2R). *Results.* Upon apoptosis induction, in Jurkat T-cell leukemia cells, the rhodamine 110 cleavage signal precedes phosphatidyl-serine externalization. No such signal is obtained in post-apoptotic cells, indicating that the cleavage of the rhodamine derived substrate measures an early and specific step of apoptosis signaling. The derivatives of D2R, (Z-DEVD)2R and (Z-IETD)2R are both applicable in live cells, and proved to predominantly measure activity of recombinant caspase-3 ((Z-DEVD)2R) and caspase-8 ((Z-IETD)2R). We analyzed the leukemia cells of nineteen patients with pediatric B-precursor ALL. Flow cytometric analysis with (Z-DEVD)2R shows a broad variation in the extend of caspase-3 activity only by cultivation in medium. Despite similar induction of cell death, a differential activation of caspase-3 by Cytarabine and Cyclophosphamide could be quantified, indicating that drug specific differences in activation of apoptosis signaling can be assessed. We could also show complete inhibition of DEVDase activity by ZVAD-fmk in primary leukemia cells. However, cell death was largely unaffected by caspase inhibition, suggesting that caspase independent cell death mechanisms are operative in drug induced leukemia cell apoptosis *in vitro*. In a xenotransplant model for human leukemia, cells were analyzed for chemotherapy induced cell death and caspase activity. Cytarabin induced caspase activity is not inhibited by ZVAD-fmk and cell death is even higher than in the mice without ZVAD-fmk treatment. In an *in vivo* experiment with the CEM cell line, ZVAD-fmk was able to reduce constitutive caspase activation and apoptosis in the NaCl treated control-mice whereas Cytarabin induced caspase activity was not affected by the pan-caspase inhibitor. *Conclusions/Perspectives.* Measurement of DEVD2R cleavage in primary leukemia cells permits detection and quantification of chemotherapy induced caspase activation *in vitro* and *in vivo*. Quantification of cellular caspase activation reveals differential induction of caspase activation by cytarabine and Cyclophosphamide. Induction of cell death by drug treatment *in vitro* is independent of caspase-3 activation. The marked heterogeneity of drug induced apoptosis signaling in primary leukemia cells permits further studies on its prognostic value and its use as treatment stratification.

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RESULTS OF THE PETHEMA ALL-96 TRIAL IN ELDERLY PATIENTS WITH PHILADELPHIA CHROMOSOME-NEGATIVE ACUTE LYMPHOBLASTIC LEUKEMIA

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Background and Aim. The incidence of ALL in the elderly is low and the outcome of these patients is poor. Only 20-25% of elderly patients are enrolled in clinical trials due to the presence of co-morbid disorders or poor performance status. For that reasons the number of published trials in elderly ALL is scarce. We present the results of treatment of Philadelphia chromosome-negative (Ph-) ALL patients ≥55 years treated with the PETHEMA ALL-96 trial. *Patients and therapy.* From 1996 to 2005, 31 patients with B or T precursor Ph- ALL ≥55 years were included in the PETHEMA ALL-96 trial. Induction therapy consisted of VCR, DNR (30 mg/m^2 d 1, 8, 15, 22), PDN, E. coli ASP (10,000 IU/ m^2 d 10-12, 17-19, 24-26) and CPM (500 mg/ m^2 d 1, 2, 29) over 5 weeks. Consolidation-1 (C1): MP, MTX (1.5 g/m^2 d 1, 28, 56), VM26 and ARA-C (500 mg/ m^2 d 14-15, 42-43). Consolidation-2/reinduction (C2): VCR, DNR, DXM, ASP and CPM. Maintenance-1: MP+MTX with monthly reinduction cycles (VCR, PDN, ASP) up to 1 year. Maintenance-2: MP+MTX up to 2 years in continuous CR. Triple IT therapy with MTX+ARAC+Hydrocortisone (14 doses over the first year) was performed as CNS prophylaxis. *Results.* 31 patients were included, median (range) age 66 [56-77] yr, 12 males. Median WBC count: 6 [1-99] ×10^9/L. Phenotype: early-pre-B: 7, common/pre-B: 17 and T: 5. Myeloid markers: 8/31 patients. Cytogenetics (26 evaluable patients): normal 11, hyperdiploid 2, hypodiploid 1, complex 9, t (4;11) 2 and other 1. Complete remission (CR) was achieved in 17/31 (55%) patients, early death occurred in 12 (39%) and 2 (6%) were resistant. With a median follow-up of 19 months, 2-yr. (95% CI) OS and DFS probabilities were 31% (0-51) and 32% (0-63), respectively. Removal of ASP and CPM from the induction therapy from 1999, due to excessive toxicity, significantly reduced the induction death rate (7/10 [70%] vs. 5/21 [24%]) (OR 7.46, 95% CI [1-41]) (p=0.019) and there was

a trend for increase in CR rate and OS probability (30% vs. 67% and 15% vs. 40%, p=0.063 and p=0.075, respectively) in those patients not receiving ASP and CPM for remission induction. *Conclusions.* Even excluding Philadelphia chromosome-positive patients, the prognosis of elderly ALL patients is poor. In our study, removal of CPM and ASP from induction therapy resulted in a significant decrease of early death and a trend for increase in CR rate and OS probability. In those patients efforts should be addressed to achieve CR with minimal toxicity and give subtype-oriented therapy in the post-remission period.

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MATURE B-CELL ACUTE LYMPHOBLASTIC LEUKAEMIA : A REPORT OF 31 PEDIATRIC CASES

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Background and Aim. The cure rate for childhood acute lymphoblastic leukaemia (ALL) now exceeds 75%. This rate has been achieved in part by improvements in the biologic characterization of newly diagnosed patients. The stage of maturation of leukaemic cells, as defined by immunology markers, has been shown to be strongly associated with treatment outcome in B-lineage childhood ALL. To demonstrate the clinical significance of a B mature phenotype, we studied the presenting clinical and biological characteristics and survival of 31 children with this phenotype. *Patients and Methods.* Thirty-one children with mature B-ALL diagnosed between 1989 and 2004 from eleven centers of the EORTC-CLG have been included in the study. All patients had blasts that express at diagnosis surface immunoglobulins (sIg) and a morphologic type of L1 or L2 (French-American-British classification). L3 were excluded. All of them were treated according to EORTC-CLG chemotherapy protocols 58881 or 58951. Characteristics of children with a mature B-cell phenotype were compared with those of the entire pediatric ALL population. *Results.* Mature B ALL was encountered in 1.1% of pediatric B progenitor ALL. Median age was 4.5 years but newborns and teenagers were more concerned. Initial leukocyte count (median 23.5 $\times 10^9$/L) was greater than 100 x 109/l in 23.4% of cases versus 14.3% in the entire ALL population. Immunophenotypically, blast cells were positive for TdT, HLA-DR and CD 34 which correspond to an early stage of precursor B-cell differentiation. CD10 was expressed in 92% of patients and in major cases sIg was an IgM lambda. Karotypic analysis was negative for 8q24 (myc) translocation, t(9;22) (q34;q11) and t(4;11) (q21;q23). Karyotypic abnormalities, confirmed by molecular studies, included t(12;21) (p13;q22) and t(1;19) (q23;p13). Of interest, hyperdiploidy was less frequent with 8.7% versus 30.3% in the entire ALL population. The results for children with mature B-cell ALL appeared poor with only 44.4% event free survival at 7 years. Many patients had high risk factors (age, leukocytosis). Unfortunately the study of minimal residual disease was not available during the 58881 protocol. It would be better to analyse the results in a prospective study including all known prognosis factors. *Conclusion.* Our data suggest that mature B-cell ALL should be considered as a new ALL entity since biological and clinical characteristics are relevant. Children with mature B-cell ALL seem to have a poor prognosis in the context of the therapy used (EORTC protocols) and the choice of more aggressive chemotherapy should be considered in this category of patients. A prospective analysis of mature B-cell ALL, in a European setting, would be of interest.

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SMALL 9P21-DELETIONS DETECTED BY MATRIX-CGH IN PATIENTS WITH ADULT ACUTE LYMPHOBLASTIC LEUKEMIA INDICATE POOR PROGNOSIS

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Background. The chromosomal band 9p21 harbours 5 different genes with potential tumor suppressor gene (TSG) function: p10, p14ARF, p15INK4B, p16INK4A and the gene for methylthioadenosine phosphorylase (MTAP). Deletions with subsequent inactivation of these TSGs are frequently observed in several malignant tumors including childhood ALL. In this type of childhood leukemia, 9p21 deletions are associated with poor prognosis. In addition, patients with adult ALL within the German GMALL study are stratified into standard, high risk or very high risk according to underlying chromosomal abnormalities. The karyotype analysis is done by standard cytogenetic procedures such as chromosomal banding. Small deletions in the range of 1-3 Mb are not detected by these methods by they might play a role for the prognosis of the affected patients. *Aim.* We analyzed the 9p21 locus by matrix CGH as well as the expression of the MTAP and p16INK4A proteins in three ALL patients with an early relapse after initially achieving a complete remission. *Methods.* Total DNA from three ALL patients (1 T-ALL, 2 cALL) was extracted using a commercially available extraction kit. Two patients relapsed either after induction phase II or consolidation II according to the GMALL 07/03 protocol. Patient 3 was initially classified as high risk due to an increased leukocyte count (32.4 G/L) and underwent allogenic PBSCT. She relapsed 8 weeks thereafter. All patients had achieved complete remission after induction phase I. Samples were analysed using a 2.8 k matrix DNA chip which contained 2800 different clones with 147 clones covering chromosome 9. In addition, the expression of the p16INK4A and MTAP proteins was examined by western blot analysis. *Results.* All three patients had small 9p21 deletions at the time when they relapsed. One patient showed a homozygous deletion and two patients a heterozygous deletion. In one patient (T-ALL), the deletion was already present at diagnosis, but could not be detected by standard cytogenetic *Methods.* In the other two patients, matrix CGH did not show any abnormalities at diagnosis. No other deletions or chromosomal gains could be identified. In addition, none of the patients expressed the p16INK4A protein, with two patients (T-ALL, 1 c-ALL) where also negative for the MTAP protein. Both genes are located closely (approx. 100 kb) to each other on 9p21. All patients were resistant to several salvage therapies and died within 3 months. *Conclusions.* Small deletions of chromosomal regions are not detectable by standard cytogenetic *Methods.* If these regions harbour cell cycle regulating tumor suppressor genes they might have a significant impact on the prognosis of the underlying disease as suggested in this study. It is therefore mandatory to include new techniques such as matrix CGH into the diagnostic toolbox, which could change the stratification strategy of risk groups in adult ALL.

0666

EFFICACY AND TOXICITY OF IDA/NOVA-FLAG REGIMEN AS SALVAGE TREATMENT FOR PATIENTS WITH ACUTE LEUKEMIA FIVE-YEAR-EXPERIENCE OF A SINGLE INSTITUTE

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Background. The observation that Fludarabine administration before Aracytine can lead to larger cytoplasmic concentrations of the latter, was the origin for the design of Ida/Nova-FLAG regimen. *Aim.* The present study aims to show that the Ida/Nova-FLAG regimen is a safe and efficacious treatment choice for patients (pts), including elderly, with refractory or relapsed AL. *Methods.* During the last five years 44 pts (32 pts<65 year-old and 12 pts≥65 year-old) with AL received in our department the Ida/Nova-FLAG regimen. Their disease was either primary resistant to chemotherapy (17) or relapsed during therapy (21) or within the first year after completion (5). The chemotherapeutic protocol included: Fludarabine: 25mg/m^2 in 1h iv infusion, 4 hours later Aracytine:3 g/m^2 (or 1g/m^2 for pts> 65 years of age) in 3h iv infusion and 1 hour later Idarubicin: 12mg/m^2 or Novantrone: 10mg/m^2 1/2 h iv infusion. *Results.* Myelosuppression with febrile neutropenia (55% of episodes with microbiological evidence and the other unspecified-FUO) was the main toxicity of the regimen. Treatment related mortality (TRM) was 22, 5%. The incidence of TRM was 50% among older pts and 20% among pts<65. Six pts died due to infection and in 4 due to hemorrhage. Two pts > 65 (16%) and17 pts< 65 (55%) obtained CR after Ida/Nova-FLAG. Eight pts with primary resistant disease (44,4%), 7 pts who relapsed during therapy (33%) and 4 pts who relapsed shortly after it (80%) were in CR after Ida/Nova-FLAG. The following table shows the response according to type of disease.

Table 1.

Disease/number of pts	CR	PR, SD	PD	Early Death
ALL (N=15)	8 (53,3%)	2 (13,3)	3 (20%)	2 (13,3)
De Novo AML (N=16)	7 (43,7%)	2 (12,5%)	5 (31,2%)	2 (12,5%)
AML from preexisting MDS (N=12)	2 (16,6%)	1 (8,3%)	5 (41,6%)	3 (25%)
SUM (N=44)	17 (38,6%)	5 (11,4%)	13 (29,5%)	7 (15,9%)

The median duration of neutrophil (>500/μl) and platelet (> 50.000/μl) recovery was 21.5 days (min: 1month, max: 72months). The TTP for pts who achieved CR was 2.5 months. Five pts (11%) received allo-BMT after Ida/Nova-FLAG, however 4 experienced relapse within the first trimester after transplantation. 13 pts received third line treatment after Ida/Nova-FLAG (e.g. Mylotarg, L-Asparaginase, Hycamptin-Ara-C, and ESHAP) but only 1 obtained CR and the other did not respond. *Conclusions.* The salvage regimen Ida/Nova-FLAG for pts with AL presents acceptable toxicity and favorable outcome, even for those with refractory ALL, for whom the data in the literature is sparse. Finally Ida/Nova-FLAG regimen can be a treatment modality for allo-BMT candidates.

0667

EFFECT OF INTENSIVE CHEMOTHERAPY ON INNATE IMMUNITY IN CHILDREN WITH ACUTE LEUKEMIA AND NON-HODGKIN LYMPHOMA

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Background. Intensive combination chemotherapy in acute leukemia (AL) and non-Hodgkin lymphoma (NHL) results in a profound systemic immunosuppression. This state, in some patients, may be responsible for recurrent and sometimes life-threatening infections. *Objective.* The aim of this study was to examine the reconstitution of the CD3-CD16+CD56+ NK and the CD3+CD8high+CD57+ immunosuppressive T cells as well as to analyze the neutrophil's and monocyte's phagocytic activity and NK cytotoxic activity in children with acute leukemia and NHL after an intensive chemotherapy. *Patients and Methods.* The study group consisted of 27 children (18 patients with acute lymphoblastic leukemia (ALL), 3 children with acute myelogenous leukemia (AML) and 6 NHL patients), aged 3 to 16 years, treated in the Department of Pediatrics, Hematology, Oncology and Endocrinology at Medical University of Gdansk. Each patient was examined 2 weeks after cessation of an intensive chemotherapy and thereafter every three months during a year. The study consisted of a medical examination, anamnesis towards infections and laboratory tests. The whole blood count, the lymphocytes subpopulations (NK cells CD3-CD16+CD56+ and the non-specific immunosuppressive T cells CD3+CD8high+CD57+) were analyzed with flow cytometry. NK cytotoxic activity was measured with colorimetric assay based on cytoplasmatic LDH activity released by damaged cells. The investigation of phagocytosis was performed by flow cytometry (ingestion of FITC-labelled opsonized E. coli bacteria by granulocytes and monocytes in whole blood was measured). *Results.* The results of our investigation indicate that: 1. The state of immunosuppression such as leucopenia remained in the patients stable during the observation time; 2. There was noticeable a tendency towards a decrease with subsequent rapid increase of cytotoxic NK response with a stable percentage and absolute number of NK cells and the immunosuppressive T cells subset; 3. The phagocytic activity of neutrophils was increased at the beginning of observations and three months thereafter it started to decrease; 4. The phagocytic activity of monocytes remained stable with a concomitant decrease of their absolute number at 12 months of observation. Only four patients (14,8%) developed severe infections after intensive chemotherapy. *Conclusions.* Intensive chemotherapy in children with acute leukemia and NHL besides severe leucopenia induces also transient changes in cytotoxic function of NK cells and phagocytic activity of neutrophils.

0668

RISK-ADAPTED THERAPY FOR ELDERLY PATIENTS WITH ACUTE LYMPHOBLASTIC LEUKEMIA

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Background. Acute Lymphoblastic Leukemia is uncommon and scantily curable in patients over 60 years of age because of a greater resistance to chemotherapy, a relative inability of elderly patients to face the toxic effects and complications of therapy and influence of co-morbidities. *Aims.* We review here our experience of 44 consecutive cases of ALL of elderly age collected in the last fifteen years. Median age was 66 years (range 61-83). L2/L1 FAB classification: 38/6; Median WBC was 15×10^9/L (range 1-180); Male/Female ratio was: 26/18. Forty cases (90,9%) belonged to B cell lineage (pre-pre-B 11, common 24, pre-B 5) and 4 (9,1%) to T cell lineage (pre-T stage); CD34 expression was observed in 27/34 cases (79,4%); CD33, CD13 and CD15 surface expression was positive in 17/35 (48,6%), 14/34 (41,2%) and 5/24 cases (20,8%), respectively; overall, CD13 and CD33 were co-expressed on 9/34 cases (26,5%). Philadelphia chromosome was present in 13 patients (29,5%). *Methods.* Out of the 44 revisited patients, 31 *younger patients* (median age 65 years, range 61-77, good performance status and without co-morbidity factors), received an intensive treatment such as LAL 0183 or 0288 GIMEMA protocols. In the remaining 13 'older patients' (median age 77 years (range 61-83) and those with severe co-existing cardiac, pulmonary, renal and hepatic disease, a *gentle* chemotherapy including prednisone and vincristine, 6-mercaptopurine and methotrexate was utilised. *Results.* Six patients (19,3%) of the group treated with curative intent died during the induction phase; 19 patients (61,3%) achieved a CR and, at present, 3 patients are alive at +10, +46 and +105 months. Out of 13 patients receiving less intensive and supportive treatment, only 4 (30,8%) achieved a short CR: all the patients had an early relapse and dead after 4, 5, 6 and 12 months. *Conclusion.* Our data demonstrate that immunophenotypic and karyotypic patterns of these patients differs from those usually observed in children and adults with ALL, therefore confirming the presence of a stem cell disorder and an extremely poor prognosis. In addition, in our experience emerged that to the 'biologically younger patients' who can well tolerate an aggressive therapy this approach should not be denied because of it is possible to achieve longer survivals.

0669

PROGNOSTIC RELEVANCE OF THE IMMUNOPHENOTYPE IN ADULTS AND CHILDREN WITH T-LINEAGE ACUTE LYMPHOBLASTIC LEUKEMIA

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Background. T lineage Acute Lymphoblastic Leukemia (T-ALL) accounts for 15-20% of newly diagnosed cases of ALL. It is characterized by a male predominance, high WBC count, mediastinal tumors and central nervous system involvement. Historically, T-ALL patients (pts) have a worse prognosis than other ALL patients. *Aims.* In this paper we review our experience on 66 consecutive pts with T-ALL (17 children and 49 adults) diagnosed and treated in our center. Median age of adults and children was 22 (range: 16-75) and 9 (range: 4-15) years, respectively. Male/Female ratio was 47/19 (adults 33/16; children 14/3) *Methods and Results.* Based on their immunophenotype, all pts were classified in 3 ontogenic stage-related subtypes: I. Early T-ALL (immunophenotype: CyCD3+/CD7±/CD1-/CD3-): 39 pts (59,1%) belonged to this group. Adult/Childhood ratio was 33/6; Median WBC was 19×10^9/L (range 1-260); in 14 pts (35,9%) a mediastinal mass was present; CD34 expression was observed in 26/34 cases (76,5%); myeloid antigens (MyAg) (CD13 and/or CD33 and/or CD15 and/or CD65) were co-expressed in 18/35 cases (51,4%). II. Cortical T-ALL (immunophenotype: CD7+/CD1+/CD3-): 20 pts (30,3%) were included. Adult/Childhood ratio was 12/8; Median WBC was 39×10^9/L (range 7-1000); mediastinal tumor was present in 13 pts (65%); CD34 was positive in 4/17 cases (23,5%) and MyAg were co-expressed in 1/16 cases (6,2%). III. Mature T-ALL (immunophenotype CD7+/CD1-/CD3+): the remaining 7 pts (10,6%) were included. Adult/Childhood ratio was 4/3; Median WBC was 18×10^9/L (range 4-480); mediastinal tumor was present in 4 pts (57,1%); none of them expressed CD34 and MyAg co-expression was only present in one case (14,3%). Therapeutic approaches applied during the twenty years period of the study were those of GIMEMA (for adults) and AIEOP (for children) cooperative groups. Overall, 51 pts (77,3%) achieved Complete Remission: (35 (71,4%) and 16 (94,1%) adult and childhood pts, respectively). Considering immunological groups, 27 (69,2%) early T-ALL, 18 (90%) cortical T-ALL and 6 (85,7%) mature T-ALL pts significantly achieved CR (p=0.035); of these, at present (median follow-up 136 months - range: 5-236), 24 pts are alive in CCR (subtype I: 10 patients (37%); subtype II: 12 pts (66,7%); subtype III: 2 pts (33,3%) p=0.012). *Conclusion.* Our data confirm that T-ALL may be quite heterogeneous in terms of clinical and biological features: a lower incidence of lymphomatous features was observed in the less mature subtypes of T-ALL, in which, in contrast, an higher co-expression of CD34 and MyAg was found. From our experience comes out that

the immunologic classification is the most significant prognostic factor in T-ALL: in fact, in adult as well as in children T-ALL, the cortical subtype showed a better outcome as compared to early and mature subtype.

0670

BONE MINERAL DENSITY IN SURVIVORS OF ACUTE LYMPHOBLASTIC LEUKEMIA DURING CHILDHOOD

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Cure rates for children with ALL now approach 80%. Therefore, the late adverse effects of chemotherapy are more frequently observed. These children are especially at high risk of developing low bone mineral density (BMD) predisposing to severe osteoporosis in adulthood. The aim of this study was to evaluate BMD and bone mineral metabolism (BMM) and the influencing factors on them. Method: We analyzed the data of 70 children who achieved complete remission with ALL-BFM protocols. Their median follow up period was 4.3 years. Children were treated according to their leukemia risk spesific groups (Standard:16, Median:45, High:9). The groups according to cessation of treatment included within one year, between 1-2 years and longer than 2 years. Their height and weight measurements and percentiles were determined both at the time of diagnosis and when they were included into the study. BMD at post-chemotherapy was also measured in lumbar area with dual X-ray absorptiometry (DEXA), and the results were expressed as age and sex-specific z scores. Serum IGF-1 and 25(OH) D-vitamin levels were measured at the time of study and the results were compared with the healthy controls. Using logistic regression test, we compared the association of BMD change with the cessation of treatment, risk groups, the cumulative steroid dose, cranial radiotherapy, passive smoking, duration of television watching and daily calcium intake. Serum IGF-1 and 25(OH) D-vitamin levels in each risk group were compared. Logistic regression analyses revealed that the most significant factor influencing BMD was daily calcium intake (OR: 0.997; 95%CI: 0.995-0.999). *Results.* The mean age of children at the time of diagnosis and study were 5.7±3.4 and 10.6±3.8 years, respectively. Percentiles both for height and weight at diagnosis and postchemotherapy increased non-significantly. The mean BMD and z score were found 0.602±0.15 g/cm^2 and -1.72±0.83, respectively at the time of study. The increase in z score values tended to correlate with the time that elapsed after the cessation of treatment, but it was non-significant. The rate of osteoporosis (z score <-2 SD) was found 44.3% whereas 41.4% of children were found osteopenic (-1<z score<-2). Cumulative steroid dose in all risk groups and cranial radiotherapy had no effect on BMD. BMD was found low in passive smokers, but it was non-significant. There was a negative correlation between BMD and the duration of television watching (p<0.01). BMD and daily calcium intake showed positive correlation (p<0.001). Serum IGF-1 and 25(OH) D vitamine levels were significantly low in ALL patients than in control group (p<0.001). However, the levels in each leukemia risk group were not different (p>0.05). Serum IGF-1 had also positive correlation with BMD (p<0.01). Logistic regression analyses revealed that the most significant factor influencing BMD was daily calcium intake (OR: 0.997; 95%CI: 0.995-0.999). *Conclusion.* BMD was determined in 85% of children. Daily calcium intake was the most significantly factor influencing BMD. Serum IGF-1 levels was also found valuable in determining the severity of osteoporosis in leukemic children.

0671

LONG TERM OUTCOME OF ALLOGENEIC AND AUTOLOGOUS STEM CELL TRANSPLANTATION IN PATIENTS WITH ACUTE LYMPHOBLASTIC LEUKEMIA COLLABORATIVE ANALYSIS FROM THREE CENTERS

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The allogeneic and autologous stem cell transplantation (SCT) are accepted treatment options for acute lymphoblastic leukemia (ALL) patients, but criteria for choosing type of transplantation remain controversial. We retrospectively evaluated long-term outcome of SCT in 68 patients with Ph neg-ALL treated between January 1995 and December 2005 from three centers in Poland. There were 39 pts, median age 24 (15-55) years, standard risk- 9 pts (23%), high risk- 30 pts (77%) in alloSCT group and 29 pts, median age 24 (17-50) years, standard risk- 12 pts (41%), high risk- 17 pts (59%) in autoSCT group. High risk patients were defined by at least one of the following criteria: leukocytosis > 30 G/L, age >35 yrs, immunophenotype pre-pre-B, early-T, CR not achieved after 4 weeks of induction. The disease status before SCT was CR1- 36 pts, CR2- 6 pts, NR- 3 pts in alloSCT group, and CR1- 27 pts, CR2- 2 pts in autoSCT group. TBI based conditioning regimens were used in 21/39 pts in alloSCT and in 8/29 pts in autoSCT group, whereas other patients were conditioned with chemotherapy: Bu/Cy2 and fludarabine/melphalan in alloSCT group; Bu/CY2, Bu/Cy/VP, .BEA and CAV in autoSCT group. CSA and a short course of MTX were given as GVHD prophylaxis after alloSCT from HLA-matched siblings (35 pts) with ATG added in SCT from MUD (4 pts). With a median follow-up of 33 (2-127) months 14/39 pts (36%) died after alloSCT, 3 in first 100 days (relapse-1, VOD-1, infection-1) and 11 after 100 days (relapse-8, GVHD- 2, infection- 1). With a median follow-up of 41 (7-81) months 13/29 pts (45%) died after autoSCT, 5 in first 100 days (infection-3, bleeding -1, relapse-1) and 8 after 100 days (relapse-8). DFS was 50% (95% CI 32-69) and 52% (95% CI 33-70) at 5 years for alloSCT and autoSCT group respectively estimated with the Kaplan-Meier method. The cumulative incidence of relapse and NRM were 33% (95% CI 19-57) and 14% (95% CI 6-32) respectively for alloSCT group versus . 35% (95% CI 35-59) and 13% (95% CI 5-32) for autoSCT group. DFS for standard risk ALL patients treated with alloSCT was 75% (95% CI 45-100) due to no relapse versus 43% (95% CI 15-71) for autoSCT, however the differences did not reach statistical significance (p 0.18), similarly as for high risk ALL pts. *Conclusions.* There were no statistical significant differences in long-term outcome between alloSCT and autoSCT for patients with Ph-neg ALL. Relapse remains the main cause of treatment failure after both allo- and autoSCT. Our results suggest that novel methods of patients stratification should be studied in further prospective clinical trials to determine which group of patients would benefit the most from each transplant option.

Chronic myeloid leukemia III

0672

CYTOGENETIC AND MOLECULAR MONITORING OF MINIMAL RESIDUAL DISEASE IN PATIENTS WITH CML IN CHRONIC PHASE ON IMATINIB MESYLATE THERAPY THE SINGAPORE GENERAL HOSPITAL EXPERIENCE

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Introduction. Imatinib mesylate (Glivec) has been demonstrated to induce good haematologic and cytogenetic response rates in patients with Philadelphia (Ph)-positive chronic myeloid leukaemia (CML). In addition the development of quantitative PCR technology has enhanced our ability to monitor response and minimal residual disease (MRD) at the molecular level. *Aim.* Over a 3-year period from 2002 to 2004, 45 patients in chronic phase CML were treated with Glivec 400 mg/day at our institution. Quantitative PCR (polymerase chain reaction) for p210 BCR-ABL transcripts was performed at regular intervals to determine molecular response. Bone marrow studies were also done to determine cytogenetic response. *Methods.* Real-Time quantitative PCR was performed on the ABI PRISM 7700 Sequence Detection System following the procedures established by the Europe Against Cancer Program. We defined major molecular response(MMR) as a 3-log reduction in BCR-ABL/ABL ratio from the median baseline of 152%, and complete molecular response (CMR) as BCR-ABL undetectable or a 4-log reduction in BCR-ABL/ABL ratio. *Results.* The median age of the patients was 45 years (range, 18-76 years) and median follow up was 31 months (range, 11-49 months). Thirty patients (67%) achieved complete cytogenetic remission (CCyR) at 12 months and on further follow-up, another 13 attained CCyR in 18-32 months (median, 24 months). Thus, a total of 43/45 (95.5%) patients were able to achieve CCyR. Among these 43 patients, 27 achieved MMR or CMR in a median of 24 months (range, 6-41 months). Twenty-three patients had subsequent PCR analyses and molecular response was sustained in 10 (43%) patients.

Table 1. Cytogenetic and molecular responses of patient cytogenetic and molecular responses of patients.

	Total	No. evaluable	No. who sustained MMR	No. who lost MMR	No. who failed to achieve MMR	Median follow-up months (range)
Total no. studied	45					
CCyR-12 months	30				10	19.5 (10-47)
MMR-12 months	5	4	3	1		16.5 (13-25)
MMR-18 months	4	4	3	1		28.5 (25-32)
MMR-24-40 months	11	9	4	5		37 (31-49)
CCyR-18-32 months	13				6	36 (25-46)
MMR	7	6	2	4		42 (34-46)

One patient lost her CCyR with 5% Ph-positive metaphases detected at 30 months and MMR achieved at 18 months was lost at 27 months. This correlated well with increasing BCR-ABL/ ABL ratios from 0.061% (18 months) to 1.071% (32 months). Of the 2 patients who did not achieve CCyR, one remained refractory with less than 0.5 log reduction in BCR-ABL transcript levels after 2.5 years on Glivec. This patient recently had an allogeneic BMT , achieved major cytogenetic response and a 1-log reduction 1 month later. Glivec was then restarted. Further monitoring of cytogenetic and molecular responses is necessary for this patient and the last patient whose latest evaluation was at 18 months. *Conclusions.* Overall, 43/45 (95.5%) of patients achieved CCyR in a median of 12 months (range, 0-32 months). This incidence rate appears to be higher than those previously reported (75%-90%). MMR or CMR was achieved in 27 patients (60% of all patients). While CCyR is sustained in most patients, molecular response is sustained in only 43% of patients. We observed that patients with fluctuating levels around 2-2.5 log reduction, remained in CCyR while a trend of persistently rising BCR-ABL transcripts could lead to a loss in CCyR. As data on molecular responses of Glivec-treated CML patients in Asia is limited, we would continue to accrue such patients for molecular monitoring to assess the association of molecular response with prolonged progression-free and overall survival.

0673

KINETICS OF TWO CO-EXISTING MUTATIONS IN THE BCR-ABL KINASE DOMAIN IN FOUR CML PATIENTS

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Background. Kinetics of mutant CML clones helps in better understanding thier functional role. *Aims.* Investigation of kinetics of co-existing mutations in CML patients *Methods and Results.* Pyrosequencing was used to study the kinetics of the mutant Ph+ clones. BCR-ABL transcripts were quantified by Taqman real time PCR. Where applicable RFLP studies were used for confirmation. Of the Ph-positive CML patients treated with imatinib (IM) at our institution who were screened for KD mutations as described previously, we were able to monitor the kinetics of the mutant clones in 4 patients, each of whom had two distinct KD mutations. Patient no. 1 failed to respond to IM and was found to have a P-loop mutation, Y253F. Treatment was changed to dasatinib, whereupon BCR-ABL transcript levels fell initially by >2 logs and the mutant clone became transiently undetectable. Thereafter transcript numbers increased and quantitative single nucleotide polymorphism (Q-SNP) using pyrosequencing and sequence analysis showed that the Ph-positive clone consisted almost entirely of mutant cells with both Y253F and T315I mutations. Q-SNP analysis suggested the two mutations were both present in 95% of cells; this was confirmed by restriction enzyme digestion of polymerase chain reaction (PCR) product. Patient no. 2 showed a similar sequence of events. She responded poorly to IM and was found to have F311L mutant clone. She responded briefly to dasatinib with transient reduction in total BCR-ABL transcripts and disappearance of the mutant clone. Thereafter BCR-ABL transcript numbers increased rapidly; the F311L mutation reappeared but a T315I mutation was also detected at the same level, namely 92%, which suggests again that the two mutations co-existed in the same sub-clone (therefore probably in cis). In contrast, patient no. 3 had no significant reduction in total BCR-ABL transcript levels despite treatment with IM. Both Y253F and M351T were detected but Q-SNP data showed that they represented on average 10% and 60% respectively of the total transcript numbers, suggesting involvement of different Ph-positive subclones. Similarly, patient no. 4 achieved complete cytogenetic remission with IM; after treatment for 1 year 80% of transcripts had a M351T mutation. After two years on IM he still had the M351T mutation (50%) but a new mutation, H396R, was detected in 46% of transcripts. Thereafter however, the levels of the two mutations evolved discordantly; for example after 30 months on IM the M351T mutation comprised 45% of transcripts whereas the H396R comprised 20%. These values imply that the two mutations involved distinct sub-clones (therefore in trans) which had different degrees of sensitivity to imatinib. *Conclusions.* These observations provide further evidence for the sequential acquisition of mutant clones, which may differ in their responsiveness to specific tyrosine kinase inhibitors. It supports the notion that the best method of preventing resistance may be to start treatment with a combination of more than one tyrosine kinase inhibitor.

0674

PROGNOSTIC SIGNIFICANCE OF THE LEVEL OF RESIDUAL DISEASE AFTER 12 MONTHS IMATINIB BASED THERAPY: THE GERMAN CML-STUDY IV

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Background. Targeted therapy with imatinib induces high response rates in chronic myeloid leukemia (CML) patients (pts). However, about 4% of pts per year relapse with reappearance of Ph chromosome positive metaphases or loss of hematologic control. *Aim.*We sought to investigate the relationship between BCR-ABL transcript levels at month 12,

cytogenetic response and relapse free survival after 2 years of imatinib-based treatment within the German CML-Study IV. *Methods.* Between July 2002 and January 2006 731 pts have been randomized of whom 251 pts were recruited until November 2003 and thereby qualified for a 2-year evaluation. In 189 pts quantitative RT-PCR data at month 12 after start of treatment are available. In parallel pts were monitored by conventional cytogenetic analysis of bone marrow metaphases. A classification of molecular response levels in 4 cohorts was applied: Ratios BCR-ABL/ABL of 0.01%, 0.12%, and 1.4% represent a 4, 3, and 2-log-reduction from a predefined baseline (IRIS definition), respectively. *Results.* After 12 mo of imatinib-based therapy, ratios <0.01% were achieved in 11 pts (cohort 1, 6%); ratios of 0.01-0.12% in 56 cases (cohort 2, 30%); >0.12-1.4% in 70 pts (cohort 3, 37%), and >1.4% in 52 pts (cohort 4, 27%). The 2-year analysis showed CCR in 7/7 evaluable pts in cohort 1 (100%), 29/29 evaluable pts in cohort 2 (100%), 33/34 evaluable pts (97%) in cohort 3, and 12/22 evaluable pts in cohort 4 (55%, $p<0.0001$). Ratios BCR-ABL/ABL after 2 years differed significantly between cohorts (cohort 1 0.011%, cohort 2 0.060%, cohort 3 0.39%, cohort 4 6.4%; $p<0.0001$). Two pts who achieved CCR at month 12 experienced cytogenetic relapse (12 and 32% Ph+ metaphases) at month 24. Their 12 mo BCR-ABL/ABL ratios were 2.5% and 2.2%, respectively, in contrast to 0.11% which represents the median ratio of those pts achieving CCR at month 12 which was ongoing at least until month 24. Taken together pts lacking CCR after 2 years (n=13, median 32% Ph+ metaphases) revealed significantly higher BCR-ABL transcript levels after 12 months than those with CCR at 2 years (13.8% vs 0.17%, $p<0.0001$). Within 2 years of observation 16/251 pts (6%) progressed to blast crisis, of whom two revealed clonal evolution (complex aberrant karyotype, n=2), and another two developed BCR-ABL kinase domain mutations, which were detectable by D-HPLC and conventional sequencing 11 mo (M244V) and 1 mo (E355G) before hematologic diagnosis of blast crisis. *Conclusions.* The assessment of BCR-ABL transcript levels by quantitative RT-PCR at month 12 of imatinib-based therapies shows prognostic significance for 2-year cytogenetic and molecular response. Long term observations will demonstrate its impact on prediction of long term response.

0675

MUTATION ANALYSIS OF THE KINASE DOMAIN OF THE BCR/ABL FUSION GENE IN CHRONIC MYELOGENOUS LEUKAEMIA

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Background. Imatinib induces complete cytogenetic remission in a high proportion of CML patients. However, patients in cytogenetic remission usually display residual bcr-abl positive progenitors by RT-PCR. The mechanisms underlying persistence of small numbers of malignant progenitors in imatinib-sensitive patients are unclear. *Aims.* To gain more information about the frequency of abl kinase domain mutations we have followed CML patients, before and during imatinib treatment, with conventional cytogenetics, qRT-PCR of the bcr/abl fusion gene transcript and mutation analysis of abl kinase domain. *Methods.* Forty-one Philadelphia chromosome positive (Ph+) CML patients, aged 23-80 years, were enrolled in the study. Thirty-one patients were in early-chronic phase (CP), 7 patients were in late-CP and 3 patients were in the accelerated phase (AP). Blood and bone marrow samples were obtained before commencing imatinib treatment, targeting a dose of 400 mg q.d. and every third months thereafter. Standard techniques were used for cytogenetic studies and qRT-PCR analysis of the bcr/abl transcript number. Mutation analysis of the bcr/abl tyrosine kinase domain was performed on samples collected before start of imatinib therapy and afterwards every 3rd to 6th month. Analysis of mutations was performed using a method modified from Shah et al (Cancer Cell 2002;2:117). Briefly, RNA was extracted from peripheral blood mononuclear cells. Complementary DNA was generated by reverse transcription followed by a first-step PCR reaction to isolate a 1.3 kb cDNA fragment which included the bcr/abl junction and the abl kinase domain. A second-step PCR reaction was performed to isolate the abl kinase domain. The resulting 0.8 kb fragment was sequenced in forward and reverse direction. A mutation was considered to be present in a sample if it was detected on both strands in two independent reactions. *Results.* After 12 months imatinib treatment, 25 patients (62%) had obtained a complete or major cytogenetic response (Ph+ <35%); 7 patients had no cytogenetic response (Ph+ >95%); 9 patients displayed resistance to imatinib defined as loss of hematologic response, loss of cytogentic response or transformation to blast crisis. No abl kinase domain mutation was detected prior to imatinib treatment. During imatinib therapy, 6 different mutations were detected in 9 patients who had shown imatinib resistance; 1 out of 31 treated in early-CP, 4 out of 7 treated in late-CP and 3 out of 3 treated in AP. E450G, a mutation locates at the C-terminal of the kinase domain distant to imatinib binding site, was the most frequently detected mutation in our material. Mutation was not detected in patients who were in hematologic remission but not having any cytogenetic response at 12 month or having residual disease detected by qRT-PCR. Some patients displayed transient mutations, even p-loop mutations, without clinical signs of imatinib resistance. *Conclusions.* abl kinase domain mutations are most frequently seen in advanced phase CML patients treated with imatinib. However, these mutations do not appear to explain the cytogenetic resistance or molecular persistent disease in patients otherwise being imatinib sensitive. Most likely mutant clones that do not expand and cause resistance, can transiently appear during imatinib treatment.

0676

BENEFIT OF IMATINIB AT 600 MG/DAY FOR PATIENTS WITH PHILADELPHIA CHROMOSOME POSITIVE CHRONIC MYELOGENOUS LEUKEMIA IN ACCELERATED PHASE: A RETROSPECTIVE STUDY OF 44 PATIENTS

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Background. Chronic myelogenous leukemia (CML) is a malignant hematologic disorder with a poor prognosis in advanced stages of the disease. Currently, the only curative approach is based on allogenic stem cell transplantation. Tyrosine kinase inhibitors and in particular Imatinib Mesylate (IM) have provided tremendous and significant improvement in chronic phase of the disease with cytognetic remissions rates above 75% in numerous studies. The benefit and the dose of IM in accelerated phase remain uncertain. Some few studies have suggested that IM at 600 mg/d could increase the cytognetic rate with a median survival close to 4 years. We report on a single and retrospective experience with IM 600mg/d in 44 accelerated phase CML patients (pts). *Patients and Methods.* 44 adult pts (M = 27, F = 17) with accelerated phase CML (IBMTR criteria) have been treated with IM at 600 mg/d. 28 pts had received a previous treatment before IM. The median time between diagnosis and IM treatment is 13,85 months (0,003-189,42). We analyzed the cytognetic and molecular responses, the overall survival and tried to determine factors possibly linked with survival. Statistical analysis have been performed with Kaplan-Meier method and the comparative curves with the log-rank method. *Results.* The median age at IM start is 51,18 years (24-80). The median follow-up time is 29,24 months. Sixteen pts died and 23 are still alive. 36 pts (84%) have a complete hematologic response, 27 (61%) a major cytogenetic response (MCyR) from whom 21 (78%) a complete response (CCyR). The probability to be in CCyR is 56% (± 17%) with a median time of 11,6 months (CI 95% : 00,0-32,7). 11 pts have a major molecular response (MMR) with a 3 log decrease of BCR-ABL load and 8 pts reached a complete molecular response (CMR). The median survival is 46,89 months. A CCyR at 3 months, and a MMR seem to be determinant for the survival (repectively $p<0,089$ and $p<0,0087$) while anemia and a previous treatment appeared to affect negatively the survival (respectively $p<0,0646$ and $p<0,029$). *Conclusion.* This study emphasize the benefit of IM at a dose of 600 mg/d in accelerated phase CML pts with substantial cytogenetic and molecular responses. A CCyR at 3 months and an MMR seem to be determinant on the progressive free survival. These results should be taken in consideration in the management of such pts particularly when the question of allogenic stem cell transplantation is raised.

0677

PRAME AS A SECONDARY TARGET FOR BCR-ABL-POSITIVE LEUKEMIAS

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Background. Chronic myeloid leukemia (CML) is a myeloproliferative disease characterized by a clonal expansion of neoplastic hematopoietic stem cell. The neogene Bcr-Abl is a hallmark of CML and it is the

result of the fusion of bcr and c-abl genes. The correlation between the bcr-abl and prame (Preferentially Expressed Antigen in Melanoma) expression has previously been suggested, but this association is still unclear. In this study, our goal was to determine the possible correlation among prame expression, Bcr-Abl levels, CML progression, and response to imatinib (Gleevec®). Aim and methodology: For this purpose we evaluate prame expression in many cell lines, such as HL-60, HL-60.BcrAbl, HeLa, HeLa.BcrAbl, Jurkat, Jurkat.BcrAbl, K562, KBM5, KBM7, KCL22, LAMA-84, SKW.64, SKW.BcrAbl, THP1, and THP1.BcrAbl (with or without imatinib for 4 hours) and 22 CML patient samples in different phases, and in remission post-imatinib by real-time RT-PCR using taqman assays. *Results.* We only found a correlation between bcr-abl and prame in HL-60 X HL-60.BcrAbl in which prame expression was 48-times higher in HL-60.Bcr-Abl. Moreover, we did not detect any association between imatinib treatment and prame, which indicates that this is probably independent of the Bcr-Abl's tyrosine kinase activity. On the other hand, a higher prame expression was related to a disease progression, as we found 8-times more prame in accelerated than in chronic phase and 29-times more in blastic than in chronic phase and no prame expression was find in cytogenetic remission post-imatinib. *Conclusions.* Recently a function of prame was described as a dominant repressor of retinoic acid receptor (RAR) signaling. Signaling through RAR induce proliferation arrest, differentiation, and apoptosis in many cell types. Considering the function and our results, we can suggest that new therapeutical approaches can be developed, aiming to inhibit the function or expression of this gene, for the most delayed phase of the illness, in imatinib-refractory patients.

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0678

GENETIC CHARACTERIZATION OF 203 *DE NOVO* CHRONIC MYELOID LEUKEMIA PATIENTS IN THE PORTUGUESE POPULATION

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Background. Philadelphia chromosome (Ph1) is the hallmark of almost all the cases of CML. The vast majority of patients express either the b2a2 (e13a2) or b3a2 (e14a2) BCR-ABL mRNA, characteristic of the p210BCR-ABL fusion protein. A very few patients express the e1a2 mRNA, characteristic of the p190BCR-ABL fusion protein and present in half of the adults who have BCR-ABL positive Acute Lymphoblastic Leukemia (ALL).[1] However, some patients have the protein p230BCR-ABL, originated from the e19a2 mRNA, and in some sporadic cases the BCR-ABL transcript is neither p210 nor p190, but another atypical product.[2,3] *Aims.* To identify the type and frequency of BCR-ABL fusion transcripts and Ph1 chromosomes in 203 Portuguese patients with *de novo* CML. Clinical diagnosis was confirmed by cytogenetics and/or molecular biology studies. *Methods.* Karyotypes were performed according to standard procedures. Molecular analyses were performed according to the BIOMED-1 Protocol.[4] 203 patients with *de novo* CML were studied (Table 1).

Table 1. Portoguese patients with the novo CML used in this study.

Patients	*Average age*	*Male*	*Female*	*Karyotype*	*Molecular Biology*
203	55.5	100 (49.3%)	103 (50.2%)	131 (64.5%)	180 (88.7%)

Results. Ph1 chromosome was found in 96.2% of patients; 3.8% were Ph1 negative BCR-ABL positive. In the Ph1 positive group 6.1% had variants [t(9;22;V), t(V;22) or t(9;V)] and 12.2% had additional anomalies, while the remaining (77.9%) presented the standard karyotype [46,XX,t(9;22)(q34;q11) or 46,XY,t(9;22)(q34;q11)]. 76% of CML patients expressed only BCR-ABL p210 transcripts, 21.2% co-expressed p210 and p190 transcripts while 2.8% expressed BCR-ABL p190 (1.7%) or b2a3 (e13a3) and e6a2, each one with a frequency of 0.56%. *Conclusions.* Our cytogenetics findings do not differ significantly from those described by other authors, except for the frequency of the Ph1 negative BCR-ABL positive cases, which is slightly below the one reported.[1,5] Based on molecular biology studies a discrepancy regarding BCR-ABL expression is shown. According to the literature more than 99% of patients express p210 transcripts, while the remaining express BCR-ABL p190 and other variants, considered rare.[1] In our population the frequency of non BCR-ABL p210 transcripts is higher than the one reported (1.7% for patients expressing p190 and 1.1% for atypical transcripts). Different transcripts may result from alternative splicing between BCR and ABL and within BCR itself. RNA splicing implies the recognition of consensus sequences, including 5' and 3' splice sites and a weakly conserved branchpoint in the intron upstream the 3' splice site. Polymorphisms affecting these sequences could activate cryptic branchpoints that are less efficiently used originating unusual products.[6] Being so, the reported frequency of atypical transcripts in our population might reflect a specific genetic *Background.* Nevertheless, the complete characterization of BCR-ABL transcripts, namely the uncommon ones, will ascertain correlations with different disease phenotypes and improve the outcome of single patients by individualizing therapeutic strategies.

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0679

GENE EXPRESSION PROFILING IN CML PATIENTS RESISTANT TO TREATMENT SPECIFIC PROFILES IN NON-RESPONDERS WITH LOW BCR-ABL TRANSCRIPT LEVELS

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CML is characterised by a presence of fusion gene BCR-ABL. The level of BCR-ABL transcript characterises the disease status and BCR-ABL kinetics are an important prognostic factor. However, we found that among patient resistant to therapy there were those whose low BCR-ABL levels did not correlate with the disease status. Moreover, patients with non-correlating BCR-ABL levels had the worst clinical outcome. Our aim was to find gene expression differences underlying this discrepancy. To do this we turn to gene expression profiling using cDNA macroarrays. We analysed 28 samples of patients not responding to treatment. There were samples with BCR-ABL levels corresponding (n=21) and not corresponding (n=7) with the clinical state of disease. Hierarchical clustering (Euclidean distance, Average linkage) was used to cluster simultaneously both samples and genes. Hierarchical clustering showed that out of 28 samples of non-responding CML patients all 7 samples with BCR-ABL level not correlating with the disease status occupied a single cluster, clearly visible on the gene expression matrix. Among gene clusters our focus was kept on genes differentially expressed in non-correlating samples compared to the rest of the non-responders. We found clusters with genes up-regulated in non-correlating samples as well as clusters with genes down-regulated in these samples. Among up-regulated genes there were BAD, CDKN2A, O-6-metylguanin-DNA metyltransferase, Notch4, RhoC and VEGFR1. Clusters of down-regulated genes included e.g. Akt2, MAPK8, cyclins A, G1 and D3 and several caspases. In conclusion, we have found a group of CML patients not responding to the treatment whose BCR-ABL transcript levels were not correlating with the clinical disease status. This group was characterised to have clearly different gene expression profiles to the other non-responding patients. The genes differentially expressed in these samples are candidates for further investigations on mechanisms of both therapy resistance and possible lose of BCR-ABL dependency in CML. The BCR-ABL independency in these patients was further supported with our preliminary data on Western blot analyses and other kinase-inhibitor experiments.

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THE EXPRESSION OF PROTO-ONCOGENES IN THE COURSE OF CHRONIC MYELOID LEUKEMIA

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Background. The chronic phase (CP) of chronic myelogenous leukemia (CML) is characterised by the presence of chimeric BCR/ABL gene and a profligate growth of mature polymorphonuclears. The accelerated

phase and blast crisis (AP and BC) of CML may show additional oncogenic aberrations and pronounced anaplasia manifested by an increase in organomegaly and blast count. The abnormal expression of some proto-oncogenes which may accompany or even precede BC of CML warrants their study. *Aim.* The follow-up of oncogene expression during the course of CML. *Methods.* We studied 85 patients (pts.) with the median age opf 50 (range 16-75 years). At the commencement of the study, 29 pts.were in CP, 25 in an AP, and 31 in the BC. The temporal expression (percentage positivity per 1000 analysed cells) of c-kit, c-myc, H-Ras, cyclin A1, p53, bcl-2 and VEGF proto-oncogene proteins over the course of CML was studied using the immunohistochemical tehnique which utilizes relevant monoclonal antibodies. It was correlated with the laboratory (Hb, WBC and platelet counts, and the percentage of blasts) and clinical parameters (organomegaly, duration of CP, AP, and BC) of disease progression. *Results.* The level of c-kit expression differed significantly in time with the largest values observed in the BC (x2, p=0,025). The level of anti-apoptotic protein bcl-2 increased significantly with the progression of CML (x2, p=0,005). Conversely, the expression of c-myc was highest in CP (x2, p=0,033). The expression of VEGF protein was most pronounced in an AP (ANOVA, p=0,033). There was no significant difference in the level of expression of H-Ras, cyclin A1 and p53 over the course of CML. The level of VEGF expression correlated inversely with degree of organomegaly (Pearson, r=-0,400, p=0,011). The c-kit expression correlated directly with the extent of bone marrow fibrosis (Spearman, r=0,407, p=0,000). High expression of VEGF correlated with a longer duration of CP (log rank, p=0,0304) and with a longer overal survival (log rank, p=0,042). *Conclusion.* The significance of changes in oncogene expression, estimated by a histochemical approach over the course of CML, may be of clinical importance in deciding on and timing of therapy. The details of the temporally-related changes in oncoprotein expression in leukemic cells require the study at the molecular level.

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P190 BCR-ABL CHRONIC MYELOID LEUKEMIA PARTLY RESEMBLING CHRONIC MYELOMONOCYTIC LEUKEMIA IN A YOUNG PATIENT TREATED WITH IMATINIB

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Background. In Ph-positive CML, the breakpoint on the BCR gene nearly always occurs within the major breakpoint cluster region (M-bcr), and the BCR-ABL fusion gene encodes a protein of 210 kDa molecular weight (P210). In most Ph-positive ALL, by contrast, the breakpoint on chromosome 22 occurs within the first intron of the BCR gene, or minor bcr (m-bcr). In these cases, the first BCR exon is fused to ABL exon 2 (e1a2 junction) and a Bcr-Abl protein of 190 kDa is formed (P190). This form of CML was reported as having some unusual clinical and haematological features, partly resembling chronic myelomonocytic leukemia (Melo *et al.*, 1994). Here we describe a 24 year-old female patient who presented in July 2005 with leukocytosis, when she volunteered as a blood donor, and was diagnosed as chronic phase CML. *Methods and Results.* She was assymptomatic, with only splenomegaly detected on physical examination. The peripheral blood examination showed a WBC count of 29.7×10^9/L, basophilia (4%), monocytosis (8%) and a platelet count of 713 ×10^9/L. No pseudo-Pelger-Huet hypolobulation or peripheral blood myeloblasts were detected. Bone marrow cytogenetic analysis at diagnosis showed a karyotype 46,XX t(9;22)(q34;q11) in 20 metaphases. Molecular studies detected the presence of an e1a2 transcript. FISH analysis confirmed the m-bcr as the sole type of BCR-ABL rearrangement present in bone marrow cells. She was put on hydroxyurea (HU) 2g daily, with a partial haematological response; 3 weeks later she was started on α-IFN 3 MU/day. Treatment with imatinib was initiated in October at a dose of 400 mg a day and after one month the dose was reduced to 300 mg daily due to moderate toxicity. The patient achieved complete haematological response and remained clinically well. After 3 months of imatinib therapy, the abnormal clone persisted and RT-PCR quantification showed a 50% BCR-ABL/ABL ratio. Despite remaining in complete haematological remission, the abnormal clone persists 4 months after initiation of imatinib therapy. Twenty-one cases of CML with a breakpoint in the m-bcr, resulting in P190 type BCR-ABL have been reported, so far, and only 17 of them in detail. This is to our knowledge, the first P190 CML case reported in a very young patient, in contrast to those previously described whose age ranged from 32 to 83 years (median 53.5) . It remains to be seen whether the long term response to imatinib in this type of CML will compare to that observed in classical P210 cases or will resemble more the poorer response achieved in Ph-positive ALL.

0682

ANGIOGENIC ACTIVATORS IN PATIENTS WITH CHRONIC MYELOID LEUKEMIA: EFFECT OF TREATMENT WITH IMATINIB MESYLATE

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Background. Angiogenesis is nowadays considered an important factor in biology of various hematological malignancies including chronic myeloid leukemia (CML). Several studies have recently reported elevated levels of angiogenic activators such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) in CML patients. However, there have been only few data on the influence of imatinib mesylate (IM) treatment on the levels of angiogenic cytokines in CML. *Aims.* To analyze peripheral blood levels of angiogenic activators in patients with newly diagnosed CML and during imatinib treatment. *Methods.* We measured plasma concentrations of VEGF, bFGF and soluble endoglin (sCD105) using sandwich enzyme-linked immunosorbent assay (ELISA) in 16 patients with chronic-phase CML and 80 healthy blood donors; furthemore, repeated samples during the therapy with (IM) were analyzed. *Results.* We found a statistically significant increase in VEGF (mean ± SD [standard deviation], 491.0 ± 365.3 vs. 64.2 ± 69.5 pg/ml, 95% CI [confidence interval] of mean, 296.4-685.7 vs. 51.0-77.5 pg/ml, p<0.0001) and sCD105 (mean ± SD, 7.0 ± 1.95 vs. 4.57 ± 1.51 ng/mL, 95% CI of mean, 5.83-8.18 vs. 4.20-4.93 ng/mL, p<0.0001) but not bFGF (p=0.606) in comparison to the control group. VEGF levels significantly decreased in 7 patients who achieved hematological remission (6 complete remissions, 1 partial remission) during therapy with IM (mean ± SD, 679.6 ± 431.5 vs. 132.7 ± 63.3 pg/ml, 95% CI, 280.6-1078.6 vs. 74.1-191.3 pg/mL, p=0.015). There was no significant change in bFGF or sCD105 (p= 0.938 and 0.125, respectively). *Conclusions.* We found significantly elevated VEGF and sCD105 levels in CML patients. In addition, successful treatment with IM resulted in significant decrease of VEGF. These data lend further support to the importance of angiogenesis in patophysiology of CML. Further studies incorporating larger number of patients are needed to confirm our findings. Supported by research project MZO 00179906 from Ministry of Health of Czech Republic

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THE E19A2 BCR-ABL BREAKPOINT: MORE FREQUENT THAN OTHER ATYPICAL BCR-ABL VARIANTS IN CHRONIC MYELOGENOUS LEUKEMIA?

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In the vast majority of patients diagnosed as having chronic myelogenous leukemia (CML) and t(9;22), the breakpoint on chromosome 22 occurs in the major region of the BCR gene (M-BCR); this translocation usually results in a hybrid BCR-ABL mRNA with a b2a2 and/or b3a2 junction, which encodes a p210 fusion protein proved to be involved in the mechanism that underlines the chronic phase of CML. Here, we report 7 newly diagnosed chronic phase CML patients with an unusual e19a2 BCR-ABL transcript. The BCR breakpoint in this type of rearrangement occurs downstream from M-BCR, in the µ-BCR region, between exons e19 (c3) and e20 (c4). This novel translocation, previously reported by our group in only few patients, results in the transcription of e19a2 type BCR-ABL fusion mRNA, which is translated into a p230-kD BCR-ABL protein. We observed that in some patients e19a2 was associated with neutrophilic leukemia while in the other patients the rare rearrangement was associated with a classical CML in chronic phase. In particular, in a 45-year-old male hemoglobin was 14.7 g/L, white blood cell count 71.8×10^9/L, neutrophils 64%, lymphocytes 8%, monocytes 2%, eosinophils 3%, basophils 5%, metamyelocytes 7%, myelocytes 9%, promyelocytes 2% and platetet count 277×10^9/L. In a 30-year-old female hemoglobin was 9.4 g/L, white blood cell count 108 ×10^9/L, neutrophils 30%, lymphocytes 4.8%, monocytes 4.3%, eosinophils 0.9%, basophils 4.2%, metamyelocytes 30%, myelocytes 20% and platetet count 9.9 ×10^9/L. In all 7 patients cytogenetic analysis of 20 bone marrow

metaphases, using G-banding, showed the t(9;22)(q34;q11) in all cells. BCR-ABL was analyzed by multiplex polymerase chain reaction (PCR), using four primers to generate PCR products from BCR-ABL and normal BCR gene transcripts. This resulted in a band of about 900 bp, in addition to the same 808-bp band representing the BCR transcript. Using two of the multiplex primers (B2B, 5'ACAGAATTCCGCTGACCATCAATAAG 3'; and CA3, 5' TGTTGACTGGCGTGATGTAGTTGCTTGG 3'), the 900-bp product was generated, indicating that the additional sequence was due to exons downstream of e14(b3). Sequencing of the PCR products after amplification with specifically designed primers revealed an in-frame BCR-ABL e19a2 transcript. It is interesting to note that in one of 7 patients there were both a b3a2 and e19a2 transcripts. We conclude that the e19a2 BCR-ABL transcript is not so rare transcript in newly diagnosed CML patients.

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GENE EXPRESSION PROFILE OF PATIENTS INNATELY RESISTANT TO IMATINIB MESYLATE

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Background. Imatinib (IM) a specific ABL tyrosine kinase inhibitor has been reported to have a significant clinic effect on chronic myeloid leukaemia (CML). Some patients treated with IM acquire resistance probably due to selective pressure on cells that carry amplified copies to of the BCR-ABL oncogene or point mutations in the ABL affecting the binding site of the drug. In others cases resistance appear to exist prior to drug exposure. Such innate mechanism of resistance is poorly understood, some evidences suggest that activation of alternative pathway, may confer BCR-ABL independent survival to CML cells. Comparative genome expression studies have long been known to provide important insight into biological process such as proliferation, differentiation, apoptosis and transformation. Only few gene expression profiling-based studies of CML and IM treatment have so far published. Moreover, only three studies has been performed on patient's samples, resulting on heterogeneous conclusions. *Aims.* To investigate about the molecular events involved in innate IM resistance in CML we compared the expression profile of a set of 380 genes on resistant patients versus responder patients. We chosen 380 genes involved in process like apoptosis, cell adhesion, cell proliferation, signal transduction, chromosome/DNA dynamics. *Methods.* A set of 13 patients (3 female, 10 male, median age 50) with CML was selected from several diagnosed at Division of Hematology of the *Cervello Hospital* of Palermo. Patients were defined as responder to IM if they achieved reduction of BCR/ABL transcript greater than 3 log within 6 months, while resistant those with less than 1 log of reduction after 6-12 months of treatment. We use the TaqMan MicroFluidic Card (Applera). This technology is a method for real-time RT-PCR that can simultaneously assays the RNA expression levels of up to 380 genes on a single card. RT-PCR data were quantified using the SDS 2.1 software and normalized using the GAPDH as endogenous control. *Results.* After the analysis of seven responder and six no responder samples we detected differential expression of 18 genes that correlate with the imatinib resistant phenotype. The resistant cells over express (1.9 fold /7.5 increase) genes of. different categories: signal transduction (SOS1,PEA15 STAT5B), apoptosis (BCL2, BAX), genes involved in cell adhesion (SELL, ITGB7), genes related to cell cycle progression (CCND2, CDK4) and transcription factors genes (ETS2, SMAD1,KLF7). *Conclusions.* In the pathogenesis of CML the expression of BCR-ABL activates multiple signalling cascades. It has been recently demonstrated that IM treatment increase BCL6 expression through the inhibition of PI3-K/AKT pathway; BCL6 replace STAT5 at STAT5/BCL6 site in CCND2 promoter repressing CCND2 expression and arresting the cell cycle progression. In this study we identified several genes implicated in cellular process that are involved in the PI3-K /Stat5 integrated mechanism, in particular we noted an over-expression in NR patients cells of STAT5B, CCND2 and CDK4 suggesting that an activation of these pathways may represent a novel mechanism for the persistence of BCR-ABL-positive cells in IM-treated patients.

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0685

PROGNOSTIC SIGNIFICANCE OF WT1 GENE EXPRESSION IN CHRONIC MYELOGENOUS LEUKEMIA TREATED WITH IMATINIB

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Background. Despite the availability of imatinib (Glivec®, Novartis, NJ, USA) as the accepted standard approach to the treatment of newly diagnosed patients with CML, the overall management of the disease has become more complex than ever. The cytogenetic and molecular response to imatinib in patients in first chronic phase overrides the currently employed pretherapeutic risk factors, i.e. Sokal score. New baseline biological markers predicting the response to imatinib therapy would thus be valuable to identify patients in whom imatinib will fail, so that timely adjustments can be made to the overall therapeutic strategy. *Aims.* The objective of this study was to evaluate if the WT1 (Wilms tumor gene 1) gene expression at diagnosis bears any prognostic information, i.e. if it relates to the cytogenetic response obtained during the first year of imatinib therapy in chronic phase CML patients. *Methods.* Peripheral blood (PB) and bone marrow (BM) samples were obtained in 25 newly diagnosed chronic phase CML patients, before commencing imatinib treatment, for analysis of WT1 gene expression. In addition, BM and PB were sampled at 3-month intervals to determine the cytogenetic and molecular response, respectively. Quantitative RT-PCR techniques (qRT-PCR) were used to measure BCR-ABL and WT1 transcript levels, and conventional karyotyping was used for measuring the cytogenetic response. Good cytogenetic responders were defined as patients having obtained a major cytogenetic response (MCgR), i.e. < 36% Ph-positive metaphases, within 1 year of treatment. *Results.* At diagnosis the seven CML patients with a suboptimal cytogenetic response were found to have significantly higher WT1 transcript levels compared to those 18 patients with a good cytogenetic response (MCgR) ($p<0.02$) This difference was seen both when peripheral blood and bone marrow samples were used as templates. No relationship was seen between BCR-ABL transcript levels at diagnosis and the cytogenetic response to imatinib therapy obtained within the first year of treatment. *Conclusion.* A high WT1 gene expression level at diagnosis might identify those CML patients that will have a suboptimal cytogenetic response to imatinib therapy. It appears warranted to study this hypothesis in a larger patient material.

0686

MONITORING OF MOLECULAR RESPONSE TO STI OR STI-INFα OF CHRONIC MYELOID LEUKEMIA (CML) PATIENTS

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Background. Despite the continuously increase control of CML obtained in the last two decades with INFα-based regimens or STI used alone or in combination with other active agents, minimal residual disease (MRD) remains detectable in the majority of patients in complete kariotypic response to this non-transplantation therapeutic strategies. *Aims.* evaluation of the molecular response rate to STI or to STI-INFα combination and the pattern of this type of remission over time. *Methods.* Thirty six Ph' positive CML patients (pts) were treated with STI at the time of diagnosis (16 pts, G1) or later in the course of their disease (20 pts,G2). Further three pts received STI-INFα combination as initial therapy (G3). This combination was also employed in eleven additional cases (G4). This group consists of complete kariotypic, but not molecular responders to early or late STI treatment (8), INFα (1) or INFα-ARA-C (2) combination. Thus, a total number of 42 patients treated with different therapies in various periods during the course of their disease, were studied. Molecular response was evaluated by RT-PCR (van Doghen JJM Leukemia: 1999, 13;1901-28) and by quantitative reverse transcriptase PCR (RQ-PCR) (*Gabert J et al. Leukemia 2003 17;2318-57*). *Results.* Molecular response was assessed in 13/16, 19/20, 3/3 and 5/11 pts of G1, G2, G3, G4, respectively. Ten pts were not evaluable because of STI (2) or INFα (3) discontinuation, patient refusal to perform the test (1), or too short treatment period (4) at the time of this analysis. A molecular response (RT-PCR) was documented at least once in 18 patients. In particular, it was documented in 7/13, 4/19 and 2/3 pts treated respectively with early or late STI or early STI-INFα combination. In 7 (3 in

G1, 3 in G2 and 1 in G3) of these 13 pts, molecular response was confirmed in two or more consecutive tests. In addition, all five evaluable pts (G4) who were in complete kariotypic response at the time of the beginning of STI-INFα combination, obtained a molecular response. This was confirmed in three or more consecutive tests over a period ranging from 15 to 23 months in three pts and in non-consecutive tests in the remaining two. From a minimum of 1 to a maximum of 10 tests (median value 4) were performed in the 18 molecular responsive patients including the first negative RT-PCR assay, in the calculation. The results obtained by RT-PCR were concordant with those obtained by RQ-PCR. *Conclusions.* It is not possible to achieve any firm conclusion regarding the effect of STI-INFα combination on molecular response because of the small sample size of treated patients. However, our findings suggest an additive effect of STI and INFα in Ph' clone control as indicated by the improvement of the quality of remission in long lasting kariotypic, but not molecular responsive patients when this combination therapy was utilized.

0687

PROLONGED MOLECULAR REMISSION IN A LATE CHRONIC PHASE CML PATIENT AFTER DISCONTINUATION OF IMATINIB TREATMENT

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Background. Treatment with imatinib results in complete cytogenetic response (CCR) in the majority of patients with Ph-positive CML in chronic phase. However, in spite of a rapid decline in BCR/ABL positive cells during treatment with a standard dosage of 400 mg/day, imatinib fails to eliminate all residual disease. It has been observed that a higher dosage of 800 mg/day may induce a more rapid CCR, and the role of highedose as well as the association of imatinib with interferon or ARA-C are under investigation in large trials, but it is not yet clear whether these more aggressive modalities of treatment will eventually lead to increased rate of response in a more prolonged follow-up. Major molecular remission (MMR), as defined by a 3-log reduction in leukemic cells, relates with good prognosis and low risk of disease progression. However, complete disappearance of the hybrid transcript, when sensitive methods such as nested RT-PCR and RQ-PCR are used, is very rare. When patients discontinue imatinib treatment because of side effects, levels of BCR/ABL rise rapidly indicating the reexpansion of the leukemic clone (Michor F *et al.,* Nature 435:1268, 2005). *Aim of the study.* To evaluate the clinical, cytogenetic and molecular features of a CML patient who obtained a complete molecular remission confirmed over a 30-months period during a standard dose imatinib treatment and persisting 22 months after imatinib discontinuation. *Methods and Results.* Standard cytogenetics, FISH analysis, nested RT-PCR and quantitative PCR with TaqMan technology were used for the follow-up of the patient. He was a 36-year-old man diagnosed as having Ph-positive CML, low Sokal risk, in 1995. No compatible donor was available. He was started on α-Interferon and subcutaneous low-dose ARA-C and went into CCR at month 18; however this was subsequently lost and a minor clone with associated trisomy 8 was observed. The patient continued IFN + ARA-C until September 2000, when BM cytogenetics showed 30% normal cells, 15% Ph+ cells and 65% Ph cells with +8. He was started on imatinib, 400 mg/day, in October 2000. A CCR was obtained at month 3 and it was confirmed in all following controls. RT-PCR and RQ-PCR became negative for the presence of BCR/ABL transcript at month 12 and this data was consistently confirmed in eight controls done every 6 months on BM and twelve PB samples, done every 3 months. In May 2004, after 43 months of imatinib treatment, therapy was stopped because of side effects. The patient remains in complete hematologic, cytogenetic and molecular remission at March 2006. *Conclusions.* Complete molecular remission as defined by the absence of any detectable BCR/ABL transcript is usually observed only in patients who underwent a allogeneic bone marrow transplantation and is uncommon in imatinib treated cases. However, the data reported here indicate that a small proportion of CML patients could achieve eradication of the disease with standard imatinib treatment. It remains to be established if the previous therapy with interferon or other immunological mechanisms may contribute to this phenomenon.

0688

CHRONIC MYELOID LEUKEMIA AFTER TREATMENT WITH 131-I FOR THYROID NEOPLASMS: TWO NEW CASES AND REVIEW OF THE LITERATURE

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Background. Chronic myeloid leukaemia (CML) is a clonal disorder arising from a somatically mutated pluripotent stem cell. It is a process of unknown aetiology, although there is a higher incidence between populations exposed to irradiation: Japanese atomic bomb survivors, radiologists, women treated for cervical cancer, and patients irradiated for ankylosing spondylitis, among others. CML after treatment with 131-I for thyroid carcinoma is a rare condition. We report two new cases of CML associated with 131-I treatment for thyroid carcinoma, and a review of the literature. *Case Report 1.* A 48-year-old female was diagnosed in February 1993 of chronic lymphocytic leukemia (CLL). She had a 2-year history of euthyroid multinodular goiter, and two years later she developed a papillary thyroid carcinoma. The thyroid gland was partially removed, and the patient began radiation therapy receiving a cumulative 131-I dose of 325 mCi, for ablation of thyroid remnants. Nine years later she developed leukocytosis and thrombocytosis, and Philadelphia (Ph) chromosome-positive CML was diagnosed. She received Imatimib as treatment, and at the present time she has only a clonal lymphocytosis in blood and bone marrow. This case is a rare combination of CLL and CML 131-I-related, and it is possible the unique known in the literature. *Case Report 2.* A 41-year-old man presented in November 2001 with a short history of weight loss, leukocytosis, and hepatoesplenomegaly. Eight years before he was diagnosed of papillary thyroid carcinoma, and received radiotherapy with 131-I, total dose 127 mCi. Typical Ph-positive CML was diagnosed. Currently, the patient is in complete remission after treatment with Imatimib. *Comment.* The leukemic proliferation of CML is related with prior X-irradiation exposure of the haematopoietic stem cell. It appears to exist direct evidence for the induction of the BCR-ABL fusion gene by X-irradiation *in vitro*. Those cells bearing this gene are positively selected by virtue of a growth advantage *in vivo*. There is a delay of several years between the initial mutational event and the development of clinical symptoms that lead to the diagnosis of CML. It was calculated that the elapsed time from occurrence of a single cell containing the Ph chromosome to a leukemic burden of 100,000 cells/μL was 6.3 years. A literature review disclosed only 10 cases similar to ours. The earliest cases of CML were diagnosed 4 to 5 years after the exposition to radiation. Although there is no evidence to prove whether the development of CML after thyroid carcinoma represents a treatment-induced complication, patients treated with 131-I may need a long-term blood count follow-up to investigate the appearance of myeloproliferative disorders such as CML.

0689

FOLLOW-UP OF CML PATIENTS WITH CLONAL CYTOGENETIC ABNORMALITIES IN PH NEGATIVE CELLS DURING TREATMENT WITH IMATINIB

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Background. Clonal cytogenetic abnormalities in Philadelphia (Ph) negative cells of patients with Chronic Myeloid Leukaemia (CML) treated with imatinib have been reported in the past years. The aberrations most frequently described are trisomy 8, monosomy 7 and deletion 20q. Few data are available regarding the outcome of such patients. We report the follow up of three patients with clonal abnormalities in Ph negative cells during treatment with imatinib. Data regarding bone marrow findings and clinical outcome are presented. *Methods.* The three patients were studied by conventional cytogenetics (CC) and by FISH with the following probes: CEP8, 5q31 (EGR-1), 7q31 (D7S486), 20q12 (D20S108) of VYSIS. All the patients had received imatinib for at least 9 months when the abnormal clone was first detected. Bone marrow was examined for morphologic dysplasia at the moment of clonal detection as well as at the moment of this study. Clinical and cytogenetic data are summarized in Table 1. *Results.* Patient 1 had myelodysplasia when the abnormal clone was first detected as well as at present. During a 26 months follow up, imatinib dosis had to be decreased because of progressive cytopenias resulting in the lost of complete cytogenetic response (CCR). Patient 2 also had bone marrow dysplasia when cytogenetic anomalies were found. CCR only lasted six months. At present, she is

in blastic phase 29 months later despite of imatinib, although never having received more than 300mg because of cytopenias. Patient 3 showed no morphologic signs of dysplasia at any moment. During a 24 months follow up he also required decreases in imatinib dosis because of cytopenias. *Conclusions.* 1.Abnormal clones were always detected in Ph negative cells. 2.The three studied patients presented cytopenias that limited increases on imatinib dosis, independently of bone marrow morphological features. 3.The correct control of the disease in these patients might be difficult because suboptimal dosis of imatinib certainly contribute to treatment failure.

Table 1. Clinical and cytogenetic data of patients when abnormal Ph negative clone was detected and at the moment of this study.

Case/ Gender/ Age	Caryotype (first analysis)	FISH (first analysis)	Caryotype (at present)	Months of follow-up / Comments
1/M/63	45,XY,-7[10]/ 46,XY [10]	-7(44%)	46,XY,t(9;22)[3]/ 45,XY,-7 [6]/ 46,XY[11]	+26/Lost of CCR. Dosis-limiting cytopenias.
2/F/64	46,XX[20]	-7(18%)	46,XX,t(9;22)[12]/45, XX,-7 [7]/ 46,XX[1]	+29/Blastic phase resistant to imatinib.
3/M/50	46,XY,t(9;22)[10]/48,XY,+Y,+8 [3]/6,XY[7]	-7(16%) +8(36%), XYY(24%)	No metaphases. FISH bcr-abl positive (3%)	+24/Lost of CCR. Dosis-limiting cytopenias.

Non-Hodgkin's Lymphoma - Clinical III

0690

THE OUTCOME OF 71 PATIENTS WITH PRIMARY MEDIASTINAL LARGE B-CELL LYMPHOMA: A SINGLE INSTITUTION EXPERIENCE

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Introduction. Primary mediastinal (thymic) large B-cell lymphoma (PMLBCL) is recognized as a separate subtype of diffuse large B-cell lymphoma with unique clinical and immunopathologic characteristics. The aim of our study was to evaluate clinicopathologic features and outcome of patients with PMLBCL. *Methods.* Between 1992 and 2005, 71 (36F\35M) previously untreated patients (pts) with PMLBCL were diagnosed and treated at our center. The median age was 36 years (range 16-66), 50 (70%) pts presented with CS IE-IIE, 58 (82%) had a bulky mass and 30 (42%) had a superior vena cava syndrome, 25 (35%) pts presented with B symptoms, LDG was increased in 55 (77%), 18 pts (25%) had IPI score 3-5. The most frequent extranodal site was lung (55%), 51% pts had pleural effusion and 25% - pericardial effusion. 52 pts (82%) received CHOP as a first-line treatment, whereas other 19 pts were treated with other regimens (9 RCHOP and 10 MACOP-B). Mediastinal radiation therapy (RT) at dose 30-36 Gy was given to 24 (34%) pts. *Results.* Among 52 pts who received CHOP, 13 (25%) achieved a complete response (CR), 11 (21%) ' partial response (PR), 7 (29%) responding pts had early relapse. All pts, who relapsed\failed the initial treatment, underwent to salvage CT, 10 not responding pts had progressive disease and died. Projected 3-years RFS and OS were 37% and 52% respectively. RCHOP and MACOP-B were highly effective regimens in all 19 pts (11 CR and 9 PR), but short follow-up and few number of pts were not indicative for the superiority of this regimens over the CHOP. *Conclusions.* Our data confirm that most patients with PMLBCL had unfavorable chances for long-term survival when treated with CHOP. While R-CHOP or MACOP-B should now be the induction treatment of choice in the majority of PMLBCL cases. A longer follow-up is needed to finally define value of these regimens.

0691

DIAGNOSTIC SENSITIVITY OF PET/CT IN PATIENTS WITH EXTRANODAL MARGINAL ZONE LYMPHOMA

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Background. 18Fluoro-2-deoxyglucose (18F -FDG) positron emission tomography (PET) is a functional imaging technique currently widely used for initial staging, follow-up and monitoring response to therapy in patients with malignant lymphoma. However, its diagnostic accuracy is overtly affected by the basic histopathology of the lymphoma subtype. Extranodal marginal zone (MALT) lymphoma comprises about 8% of all NHL cases and is the third most common type of NHL. There is a controversy in the reported literature regarding the accuracy of 18F -FDG avidity in MALT lymphomas. While earlier studies suggested a limited role for PET in MALT patients due to their non-FDG avidity, a more recent report has suggested that this is incorrect. Recently, the technique of PET imaging was upgraded by using hybrid systems composed of PET and CT in the same framework. The fused functional-anatomic data appears to provide better localization of lesions, differentiating physiologic from malignant uptake and detection of unexpected lesions otherwise overlooked. As yet no study investigating the role of PET/CT in MALT lymphoma has been reported. We hypothesized that since MALT may originate in organs with physiologic FDG uptake such as the gastrointestinal tract, the use of PET/CT may improve the diagnostic accuracy of 18F -FDG assessment. *Aims.* To evaluate the diagnostic sensitivity of PET/CT in patients with MALT lymphoma and to assess its reliability in staging and monitoring disease activity. *Methods.* Thirty patients with biopsy proven MALT lymphoma in 33 sites, who underwent PET/CT at diagnosis, were included. Medical records, PET/CT findings and data obtained by other diagnostic procedures including gastroscopy were reviewed. *Results.* Common sites of MALT were the stomach (17), orbit (4), lung (2) and parotid (2). PET/CT detect-

ed active disease in 16 of 30 patients (53%). Sensitivity in gastric MALT (7/17, 41.1%) was lower compared to non-gastric MALT (9/13, 69%). The CT findings obtained from the fused PET/CT data allowed differentiation between physiological and pathological FDG uptake, especially in the stomach. PET/CT detected active disease in 7/7 (100%) patients with advanced disease (stage III-IV) but was positive in only 9/23 (39.1%) with early stage disease (I-II). The incidence of gastric FDG uptake was higher in patients presenting with gastric ulcer than in subjects with minimal or no macroscopic findings on gastroscopy. Of the 30 patients in the study cohort, nine had a follow up PET/CT after therapy. Of these, 5 had biopsy proven relapse during follow-up. PET/CT detected relapse in 3 patients (including one patient who had negative PET on diagnosis). *Conclusions.* We report the initial results of a PET/CT imaging in MALT lymphoma patients. Our data suggest that PET/CT is a useful tool for both initial staging of disease and for follow-up after therapy. The anatomic data obtained from the CT part of the study allows for better interpretation of the corresponding scintigraphic abnormality detected on PET, mainly since MALT appears to involve organs which may be associated with physiologic 18F -FDG uptake.

0692

BENIGN STRICTURES FOLLOWING TREATMENT FOR PRIMARY GASTRO-INTESTINAL NON-HODGKINS LYMPHOMA: CASE SERIES

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Background. We report a novel complication, benign intestinal strictures, developing after treatment for gastrointestinal lymphoma (GINHL). Between 25 and 35% of non-Hodgkin's lymphomas arise at extranodal sites, and around half of all extranodal lymphoma is in the gastrointestinal tract, the stomach being the commonest site. Treatment for GINHL carries a risk of internal haemorrhage, and perforation of an abdominal viscus, although studies have found that these complications are relatively rare, with incidence rates of around 0-2%. *Methods.* This is a retrospective case series. Five patients in whom a stricture complicated therapy for lymphoma were identified from the centre records over a six year period. Information was gathered from clinical records, radiology and stored histology samples. Available histology was reviewed by an independent histopathologist and x rays were reviewed by an independent radiologist.

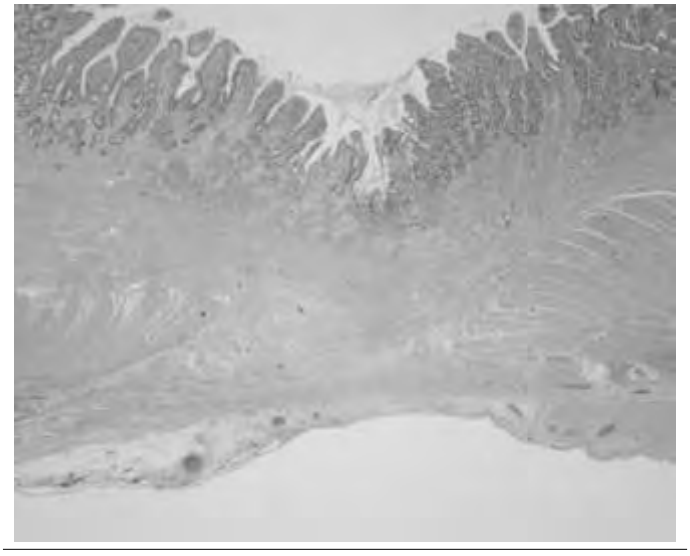

Figure 1. Duodenal stricture with replaced muscularis propri.

Results. Three patients were male and two female. The median age was 55 (range 34-75). Three patients had localised diffuse large B-cell lymphoma of the small intestine (all in the duodenum or jejunum). One patient had post-transplant lymphoproliferative disorder affecting the small bowel and another follicular lymphoma involving the gastro-duodenal junction. In 3 cases, symptoms of obstruction developed during chemotherapy and in the other 2 this occurred during remission at 10 months and two years from the end of therapy, respectively. One case was treated with CHOP (cyclophosphamide, doxorubicin, vincristine and prednisolone) (having previously received rituximab at presentation), and the remainder were treated with R-CHOP (rituximab with CHOP)/RCEOP (rituximab with etoposide in place of doxorubicin). None of the patients had surgery or radiotherapy prior to the development of their bowel stricture. In all cases histology from resection of the stricture showed only fibrous adhesions but no malignant infiltrate (an example is shown in the attached figure). All the patients remain in complete remission a median of 21 months (range 9-60) following resection, with no recurrences. *Summary/conclusions.* This complication has not been reported before. Whilst development of symptoms of bowel obstruction in patients with a previous history GINHL should always prompt a search for recurrence of the original disease, we have shown that this may also be due to the presence of a benign stricture at the original disease site, that this can be treated effectively with surgery, and with no apparent risk of recurrence.

0693

ACTIVITY OF RITUXIMAB PLUS CYCLOPHOSPHAMIDE, DOXORUBICIN/MITOXANTRONE, VINCRISTINE AND PREDISONE (R-CHOP/R-CNOP) IN PATIENTS WITH RELAPSED MALT-LYMPHOMA

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Background. Various chemotherapeutic agents as well as the anti-CD20 antibody rituximab (R) have been tested in patients with MALT lymphoma, but no standard chemotherapeutic regimen has emerged so far. Judging from data obtained in various types of lymphoma, the activity of R appears to be enhanced by combination with chemotherapy. As no data on this topic exist in MALT-lymphoma so far, we have analysed our experience with R plus cyclophosphamide, doxorubicin/mitoxantrone, vincristine and prednisone (R-CHOP/R-CNOP) in patients with relapsed MALT lymphoma. *Patients and Methods.* A total of 26 patients were treated , 15 were administered R-CHOP while 11 patients were given R-CNOP due to age >65 years or pre-existing cardiac conditions. In total, 13 patients presented in first relapse, 10 in second relapse, while the remaining 3 patients were given R-CHOP/CNOP in third relapse. A total of 16 patients (61%) had received at least one chemotherapeutic regimen before application of R-CHOP/R-CNOP, three had been pretreated with R, while various types of treatment including radiation, HP-eradication and surgery had been used for treatment before R-CHOP/CNOP. Cycles were repeated every 21 days, and restaging was performed after four cycles of therapy. In case of complete remission (CR), two further cycles were administered for consolidation while patients achieving partial remission (PR) or stable disease (SD) after restaging were given four further courses. *Results.* A total of 170 cycles were administered in our patients (median 6, range 2-8). Five of 26 cases (19%) had plasmacytic differentiation before initiation of therapy. Genetic aberrations specific for MALT-lymphoma were detected in 14 patients (54%). A high response rate was seen in our patients, as 19/26 (73%) achieved a CR and 7 patients (23%) had a PR, for an overall response rate of 100%. Response to treatment was not influenced by prior therapy, genetic aberrations or plasmacytic differentiationSide effects were mainly haematological, with 8 / 26 patients (31%) developing leukocytopenia/granulocytopenia WHO grade III/IV. This was complicated by infection in a total of three patients (fever without a detectable infection in 2 and pneumonia requiring hospitalization in one patient). All eight patients were given prophylactic G-CSF for the following cycles, resulting in application of further chemotherapy as planned. Two patients developed thrombocytopenia WHO grade III. After a median follow-up of 19 months (range; 12-45), all patients are alive: 22 in ongoing remission, while 4 have relapsed 12-19 months after treatment. All relapses were histologically verified, and two of these patients showed plasmacytic differentiation upon relapse which had not been present before initiation of therapy with R-CHOP. *Conclusions.* This is the first analysis of a combination regimen including R in the treatment of patients with MALT lymphoma. Taken together, our data demonstrate that R-CHOP/CNOP is highly active and safe even in heavily pretreated patients with MALT lymphoma.

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INFLUENCE OF RITUXIMAB, CYCLOPHOSPHAMID, DOXORUBICIN, VINCRISTINE AND PREDNISOLONE (R-CHOP) ON SEROLOGIC PARAMETERS AND CLINICAL COURSE IN LYMPHOMA PATIENTS WITH AUTOIMMUNE DISEASES

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Background. The development of a Non-Hodgkin's lymphoma (NHL) is one of the most serious complications in patients with autoimmune diseases. Mucosa associated lymphoid tissue (MALT) lymphoma and diffuse large B-cell lymphoma (DLBCL) are the most common subtypes in these patients and chemotherapy is the therapy of choice in this setting. The combination of cyclophosphamide, doxorubicin, vincristine, prednisolone and rituximab (R-CHOP) seems to be the most effective regimen for lymphoma cell eradication at the moment. On the other hand, B lymphocytes are not only the key target in NHL but play also an integral part in the pathogenesis of autoimmunity. In keeping with these findings, one might hypothesize that immuno-chemotherapy administered to treat lymphoma might also diminish (or even eradicate) auto-reactive cell clones and might therefore improve the underlying autoimmune condition. *Aims.* As patients with B-cell lymphomas suffering from an underlying autoimmune condition undergoing therapy with R-CHOP offer the unique possibility of monitoring effects of therapy on various rheumatologic parameters, we have evaluated serologic autoimmune-markers and the clinical outcome of patients with autoimmune-diseases who received lymphoma treatment with R-CHOP during the course of their disease. *Patients and methods.* We have retrospectively analysed 13 patients with Non-Hodgkin's lymphoma who concurrently suffered from autoimmune diseases and were treated with the R-CHOP regimen (Table 1).

Table 1. Patient characterists.

No.	Sex Age	Lymphoma	Rheumatic Disease	RF	ANA	C3c	C4	RD prior to NHL	RD Duration	Immunomodulatory Mediaction prior to Lymphoma Diagnosis
1	M-89	MALT	RA	neg	pos	n.d.	n.d.	no	-	none
2	F-60	MALT	SS	pos	pos	norm	norm	yes	5a	none
3	F-54	MALT	DM	pos	pos	norm	norm	yes	6a	CSA
4	M-78	MALT	RA	pos	pos	norm	norm	yes	13a	Gold, Leflunomide
5	F-84	MALT	RA	pos	pos	norm	norm	yes	1a	Steroids
6	F-73	MALT	RA	pos	neg	norm	norm	no	-	none
7	F-87	MALT	Atopy	neg	pos	norm	norm	yes	8a	none
8	F-71	DLBCL	RA	pos	pos	n.d.	n.d.	yes	30a	none
9	F-78	DLBCL	RA	pos	pos	n.d.	n.d.	yes	8a	MTX, Steroids
10	F-67	MALT	Sharp-Syndrome	n.d.	pos	norm	norm	yes	8a	Chloroquine
11	F-69	MALT	SS	neg	pos	n.d.	n.d.	yes	1a	None
12	F-69	DLBCL	SLE	neg	pos	norm	norm	yes	12a	Gold, Chloroquine, Steroids, CSA
13	F-68	DLBCL	Vasculitis	pos	neg	elev.	redu.	yes	14a	Steroids, MTX

No: number; M: male; F: female; MALT: mucosa associated lymphoid tissue lymphoma; DLBCL: diffuse large B-cell lymphoma; RA: rheumatoid arthritis; SS: Sjögren's Syndrome; DM: dermatomyositis; SLE: systemic lupus erythematodes; RF: rheumatoid factor; ANA: ant-nuclear antibody; C3: complement 3; C4: complement 4; RD: rheumatic disease; NHL: non-Hodgkin's lymphoma; CSA: cyclosporine A; MTX: methotrexate.

At every visit, patients were asked for the presence of joint pain, intake of corticosteroids, disease modifying anti-rheumatic drugs (DMARDs) and/or non-steroidal anti-rheumatic drugs (NSAIDs) as well as quality of life. The rheumatoid factor (RF), antinuclear antibodies (ANA) and the complement factors C3c and C4 were measured immediately before institution of chemotherapy and then at regular intervals during the course of chemotherapy. Lymphoma response to treatment was classified according to the International Working Group recommendations. *Results.* The median levels of RF were 189 IU/ml (IQR: 12-960) before and 65.5 IU/ml (IQR: 20.25-577.75) after therapy (p=0.046). The median levels of ANA were 240 (IQR: 70-1600) before and 40 (40-160) after therapy (p=0.068). 10 (77%) patients showed clinical improvement of their autoimmune symptoms during the course of chemotherapy. Two (15%) patients reported no difference with regard to their autoimmune disease and one (7%) patient who suffered from rheumatoid arthritis experienced a worsening of her symptoms during therapy with R-CHOP. The autoimmune related symptoms recurred after a median time of 7 weeks (IQR: 6-8) in 7 patients. One patient who had suffered from vasculitis before initiation of had a durable remission after completion of R-CHOP now ongoing for 8 months. In terms of lymphoma-response, 11 patients achieved a complete- and 2 a partial remission. *Summary/Conclusions*.This analysis suggests that R-CHOP given for lymphoma treatment is also effective for therapy of concurrent rheumatoid diseases. Both rheumatoid parameters as well as clinical symptoms showed a significant decrease during treatment with this immunochemotherapy. However, the effects on the rheumatic diseases seem to be of limited duration. With regard to the lymphoma, R-CHOP displayed an excellent efficacy which seems comparable, or even better, to patients with NHL without autoimmune diseases.

0695

PET/CT DIAGNOSTIC ACCURACY IN LYMPHOMA

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Background. Staging accuracy plays an important role in lymphoma patients to choose appropriate therapy. In the past staging algorythms have varied but computed tomography (CT) remains the imaging technique of choice although it is based on anatomic size. Positron emission tomograpy with 2-deoxy-2- (fluorine-18)-D-glucose (FDG) provides usefull functional information but its main drawback is the lack of anatomic landmarks. *Aim.*We have prospectively compared the accuracy of lesion detectability and anatomical staging of whole body PET/CT (either with full dose ehnanced contrast CT or non enhanced low dose CT) with PET and conventional staging work-up for NHL and HD. *Methods.* 47consecutive patients were included. 30 female and 17 male (mean age 50y, range 23-83y). 31 patients had NHL (1 Burkitt L,1 ACL, 1SLL, 6 MZL, 2FL, 1MCL, 19 DLBCL) and 16 patients had HD. All underwent conventional staging studies including iliac crest bone marrow biopsy and PET/CT (full and low dose). *Results.* 1) Nodal and extranodal involvement: Total agr eement was found between low dose PET/CT and full dose PET/CT for the cervical region (Kappa=1, p<0.001) and almost complete for the thoracic and abdominal region. Agreement between PET/CT and CT was found in 89% patients for the cervical region; 83% patients for the thoracic region and 91% patients for the abdomen-groin region. Globally PET/CT showed less number of indeterminate *Results.* PET/CT demonstrated 78% of patients with positive extranodal disease whereas CT only detected 61.7%. 2) Staging: Globally, agreement in staging was statistically significant among the gold standard staging algorithm (using full dose contrast enhanced CT) and the 3 new staging algorithms (PET kappa=0.54; low dose PET/CT kappa =0.624; High dose PET/CT kappa 0.628) with an almost perfect agreement between low and high dose PET/CT (kappa=0.92 p<0.001). Both algorithms with PET/CT showed statistically significant higher stages than the gold standard (McNemar test, p=0.012). 10 NHL patients were upstaged (7DLBCL, 1ACL, 2FL) all due to extranodal findings (confirmed either by biopsy in 2 cases or follow up in 7 cases, 1 case deceased before follow up). 1HD patient was upstaged confirmed by follow up. Change in staging resulted in different treatment strategy in 7 patients (14.8%). *Conclusions.* 1.PET/CT is a promising image diagnostic tool that provides a complete body survey in a disease with unpredictable recurrence that is lymphoma. 2.The kappa statistic revealed excellent agreement between low dose non enhanced PET/CT and full dose enhanced PET/CT for lymph node detection and staging. 3. Accurate lesion localization can be achieved with low dose PET/CT thus allowing a simplify and less expensive diagnostic imaging in patients with lymphoma4. PET/CT seems of great value in agressive NHL, some indolent subtipes (FL) and HD patients to provide a baseline for later response evaluation.

0696

EFFICACY AND SAFETY OF DEPOCYTE (LIPOSOMAL CYTARABINE) IN PATIENTS WITH CENTRAL NERVOUS SYSTEM INVOLVEMENT FROM NON-HODGKINS LYMPHOMA: A REPORT ON 23 PATIENTS TREATED IN SPAIN

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Background. Lymphomatous meningitis (LM) occurs in ~5% of patients with diffuse large B-cell lymphoma (DLBCL), and more frequently in patients with Burkitt's lymphoma (BL) and lymphoblastic lymphoma (LL). Conventional treatment of LM involves multiple (2-3 x weekly) intrathecal injections of cytarabine or methotrexate, which increases the burden on patients, carers and medical providers. As very few long-term survivors have been reported in any patient series, the major goals of therapy are relief from neurological symptoms, prevention of neurological progression, and preservation of quality of life. DepoCyte® is a sustained-release formulation of cytarabine for intrathecal injection, which maintains therapeutic concentrations in the cerebrospinal fluid (CSF) for 2 weeks. Treatment with DepoCyte® does not require an Ommaya reservoir, and offers the advantage of less frequent injections and potentially greater efficacy than conventional treatment. *Methods.* We report here on a series of 23 patients (median age 43 years, range 21-74; 13 male) with LM (9 relapsed) from 17 Spanish hospitals who were treated with intrathecal DepoCyte® (mean 3.5 injections, range 1-9) from March 2004 to December 2005. Concurrent dexamethasone was given as prophylaxis for arachnoiditis. *Results.* Cytological responses (clearance of lymphoma cells from the CSF) were seen in 8 of 12 patients with DLBCL; 3 of 3 with BL; 1 of 1 with LL; 1 of 2 with mucosa-associated lymphoid tissue (MALT) lymphoma; 2 of 2 with follicular lymphoma (FL); 1 of 1 with primary central nervous system lymphoma (PCNSL); and 1 of 2 with T-cell non-Hodgkin's lymphoma (NHL). Neurological responses were seen in 8 of 12 patients with DLBCL (6 complete remissions [CR], 2 stable disease [SD]); 2 of 3 with BL (1 CR, 1 partial remission [PR]); 1 of 1 with LL (PR); 1 of 2 with MALT lymphoma (CR); 2 of 2 with FL (2 CR); 1 of 1 with PCNSL (SD); and 2 of 2 with T-cell NHL (1 PR, 1 SD). The overall response rate was 74% for both cytological response and neurological response (43.5% CR, 13% PR, 17.5% SD). Neurological progression occurred in 83% of patients, including 9 of 12 patients with DLBCL, after 7-330 months (6 remained alive at last report); 3 of 3 with BL after 28-90 months (2 alive); 1 with LL after 30 months (died); 2 of 2 with MALT NHL after 8 and 95 months (died); 2 of 2 with FL after 150 and 300 months (both alive); 1 of 1 with PCNSL (died); and 1 of 2 with T-cell NHL at 20 months (alive); 48% of patients remained alive at the last report. DepoCyte® showed good tolerability. Fifty-two per cent (12/23) of patients experienced no side effects, and the most common side effects associated with DepoCyte® injection were headache (n = 8), vomiting (n = 4) and nausea (n = 3), with one occurrence each of fever and neurological deficits. *Conclusions.* This series demonstrates the feasibility, efficacy, safety and tolerability of DepoCyte® in the treatment of LM associated with different histological subtypes of NHL. A substantial number of patients had a cytological response (74%) or a neurological response (74%), and 48% of patients were still alive at last report. Two-weekly (or monthly) DepoCyte® injections are much more convenient than the conventional alternatives. We consider that DepoCyte® may be the agent of choice for LM.

0697

LACK OF HUMORAL RESPONSE TO ACUTE EBV INFECTION MAY IDENTIFY PATIENTS WITH FULMINANT EBV-ASSOCIATED NK/T-CELL LYMPHOPROLIFERATIVE DISORDER

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Background. EBV associated NK/T-cell lymphoprolifrative disorder is a rare and distinct clinical entity. In most instances, it is refractory to conventional treatment and confers a poor prognosis. We report the clinico-pathologic features of 7 patients with EBV-associated NK/T-cell lymphoproliferative disorder treated between 2002 and 2005 in a single institution. *Aims.* We compare the presenting features and treatment outcomes of these patients. In doing so, we hope to identify trends that may help improve the diagnostic and management approaches of a rare and fatal disease. *Methods.* The investigation is a retrospective study. Patients with the diagnosis of interest were identified from our lymphoma registry and clinical information of the cases obtained from pathological reports and clinicians. *Results.* All patients were of Asian origins (6 Chinese and 1 Malay). Other than a patient aged 66 years old with a history of renal transplant and was on immunosuppression, the other 6 patients were aged between 32 to 40 years with no significant past medical history. All patients had a preceding history of acute upper respiratory tract infection prior to their dramatic presentation. They demonstrated the haemophagocytic syndrome with severe systemic symptoms including high fever, acute pancytopenia, mixed cholestatic/hepatitic transaminitis and coagulopathy. Five patients had maculopapular rashes. Other than the finding of mild hepatosplenomegaly, no bulky diseases or significant lymphadenopathy were found on CT scans of all patients. LDH and β2 microglobulin were invariably elevated. Diagnoses of EBV associated lymphoproliferative disorder were made based on demonstration of in-situ hybridization for EBV-encoded early small RNA (EBER) on bone marrow trephine, skin and liver biopsies. Three patients demonstrated the classic NK/T-cell lymphoma, nasal type phenotype (CD3-,CD4-,CD8-,CD56+), with clinical manifestation of aggressive NK-cell leukaemia at the outset, with no prior history of nasal disease. Four had peripheral T cell lymphoma phenotype with one showing TCR γ rearrangement. PCR for EBV DNA performed in 3 patients showed high viral loads. Despite features of acute EBV infection, EBV capsid Ag IgM was surprising negative in all patients, while IgG was positive in all. Median survival was 53 days from time of presentation and the causes of mortality included liver failure, neutropenic sepsis and severe bleeding. Four patients received chemotherapy. Two had CHOP regime upfront and two had immunosuppresion with etoposide, prednisolone and cyclosporin prior to full dose chemotherapy. Although mortality was uniform, those who received immunosuppression first had a longer survival. *Summary/Conclusions.* This disorder should always be suspected when patient presents with haemophagocytic syndrome. Its epidemiological predisposition may be accounted for by a higher prevalence of EBV infection and carrier status in the Asian population. Important pitfall in diagnosis is the lack of serological evidence of acute EBV infection. This lack of humoral response may be the predisposing factor for clonal T-cells proliferation post-EBV infection. Reasons for this immune defect in a predominantly young and seemingly well adult population remains elusive. Immunosuppression may have a role in controlling the cytokine storm before chemotherapy is started.

0698

DOSE-DENSE CHOP (CHOP-14) PLUS RITUXIMAB FOR NEWLY DIAGNOSED AGGRESSIVE B CELL LYMPHOMA. A PROSPECTIVE MULTICENTRIC STUDY. GRUPO PARA EL ESTUDIO DE HEMOPATIAS MALIGNAS - GALICIA

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Background. Standard front line therapy for aggressive B cell lymphoma is CHOP every 21 days associated with rituximab; CHOP every 14 days (CHOP-14) has also shown an improvement in response and survival rate over CHOP-21. *Aims.* To evaluate the feasibility and efficacy of CHOP-14 plus rituximab in newly diagnosed aggressive B cell lymphoma. *Methods.* Patients (pts) 18-75 years old with *de novo* diffuse large B cell (DLBC) or follicular grade 3 lymphoma diagnosed between April/03 and December/05 received CHOP-14 in combination with rituximab (375 mg/m^2) on day 1, and with G-CSF support; a maximum of 8 cycles and radiotherapy for localized or bulky disease were planned. Forty-four pts. from six institutions have entered the study; results from 43 evaluable pts (39 DLBC and 4 follicular) are reported. Median age: 62 y (21-74), 22 pts over 60 y. Ann Arbor stage: I-II 51.2%, III 7%, IV 41.%. B symptoms: 34.9%. Extranodal involvement: 58.1%. Bulky disease: 41.8%. International Prognostic Index: low/intermediate-low 62.8%, intermediate-high/high 37.2%. *Results.* Response: 36 pts. obtained a complete response (CR) (83.7%) and 4 pts. a partial response (PR) (9.3%); 3 pts. were not evaluable for response because of early death (7%). Medi-

an n° cycles/pt: 8 (2-8), and 14 pts. received radiotherapy. Toxicity: 29/298 cycles (9.7%) were delayed, mainly because of infectious complications (17 cycles) or neutropenia (4 cycles); in 14 / 298 cycles (4.7%) the pt. required hospitalization, and all but one of these pts. were over 60 y. Hematologic toxicity: Anemia grade II - III WHO 23.3% and neutropenia grade III - IV WHO 23.3%; 10 pts. received an erithropoietic factor with improvement. Infectious complications grade III - IV WHO appeared in 14 / 298 cycles (4.7%); two pts. died from a bilateral interstitial pneumonitis without severe neutropenia and microbiological identification; none of them had received SMX-TMP prophylaxis. Other two pts. died after 2° cycle of chemotherapy from cardiovascular disease. All toxic deaths were in pts. over 65 y. Relapse: 2/36 pts (5.5%) with a median follow-up of 20 months (3-34). Survival: Seven pts. have died; 4 toxic deaths, another 2 during second-line treatment after a PR and one non-related death. The 2-years overall survival is 80.3% and progression-free surival (PFS) for pts. in CR is 79.7%. *Conclusions.* CHOP-14 associated with rituximab obtains a high rate of CR (83%) that also seems to be long-lasting, with only two relapses in 20 months and a 2-year PFS of 80%. Toxicity appears reasonable and affecting specially older pts.; only 9.7% of cycles has been delayed and, since SMX-TMP prophylaxis was initiated, no other pneumonitis have been recorded.

0699

RITUXIMAB AND ESHAP PLUS G-CSF AS AN EFFECTIVE PERIPHERAL BLOOD PROGENITOR CELL MOBILIZATION REGIMEN IN PRETREATED B-CELL NON-HODGKINS LYMPHOMA: A PRELIMINARY REPORT OF COMPARISON WITH ESHAP PLUS G-CSF

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Background. The ESHAP has been reported as excellent mobilization chemotherapy in patients with relapsed and poor-risk aggressive non-Hodgkin's lymphoma (NHL). Rituximab added to ESHAP (R-ESHAP) has been tried as salvage therapy for relapsed and poor-risk B-cell NHL. Mobilizing stem cells following R-ESHAP should decrease time to autologous stem cell transplantation (ASCT) by making separate mobilizing chemotherapy unnecessary, while controlling a patient's lymphoma. *Aims.* The aim of this study was to prospectively evaluate the efficacy of mobilization by R-ESHAP plus G-CSF regimen in relapsed or poor-risk B-cell NHL. *Methods.* Twenty B-cell NHL patients were enrolled. R-ESHAP plus G-CSF (Neutrogin®, Choongwae Pharma Corp., Seoul, Korea) was used to mobilize peripheral blood progenitor cells. The results were compared with those of 24 patients with NHL whose mobilizing chemotherapy was ESHAP. *Results.* The R-ESHAP and ESHAP groups were well balanced for age, sex distribution, prior chemotherapy cycles, number of chemotherapy regimens, and radiotherapy to the axial skeleton. Total duration of G-CSF administration was not different between the two groups. The median number of total CD34+ cells harvested per patient was 10.59×10^6/kg (range, $4.88\text{-}52.55\times10^6$/kg) in the R-ESHAP group and 15.34×10^6/kg (range, $0.04\text{-}48.0\times10^6$/kg) in the ESHAP group (p=0.42). The median number of $CD34^+$ cells collected per apheresis was 4.30×10^6/kg (range, $0.30\text{-}21.60\times10^6$/kg) in the R-ESHAP group and 4.40×10^6/kg (range, $0.01\text{-}26.50\times10^6$/kg) in the ESHAP group (p=0.71). Adequate collection (total harvested CD34+ cells > 2×10^6/kg) was achieved in all 20 patients from R-ESHAP group and 22 of 24 (92%) patients from ESHAP group (p=0.19). Optimal collection (total harvested $CD34^+$ cells > 5×10^6/kg) was attained in 95% (19/20) of patients in the R-ESHAP group and 92% (22/24) of patients in the ESHAP group (p =0.67). Kaplan-Meier product limit estimate and log-rank test revealed that the apheresis days to adequate and optimal CD34+ cell collection were not statistically different between the two groups. Thirteen patients from R-ESHAP and 19 patients from ESHAP group underwent ASCT and there were no differences in days to neutrophil engraftment and platelet engraftment. *Summary/Conclusions.* These preliminary results indicate that R-ESHAP plus Neutrogin® is an excellent mobilization regimen in patients with relapsed and poor-risk B-cell NHL.

0700

THE OCCURRENCE OF CNS RELAPSES IN HIGH-RISK AGGRESSIVE LYMPHOMA PATIENS TREATED WITH INTENSIFIED INDUCTION AND HIGH-DOSE CONSOLIDATION PROTOCOLS OF THE CZECH LYMPHOMA STUDY GROUP

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Backround. CNS relapse of systemic NHL is usually fatal and identification of risk group and effective prophylaxis are contraversial issues. *Aims.* to analyse our cohort of high risk NHL patients in terms of CNS relapses and identify risk factors for CNS relapse. *Patients and Methods.* We analysed a cohort of 135 patients younger than 65 years with high-risk (age adjusted IPI 2,3) aggressive lymphomas (73% DLBCL, 15% mediastinal DLBCL, 5% peripheral T-cell, 2% mantle cell, 1% anaplastic large cell, 4% others with no burkitt and no lymphoblastic lymphoma). Patients were prospectively treated with intensified induction and high-dose consolidation protocols designed by CLSG in the period 1998 - 2004. Treatment protocols. Protocol 1: induction 3-4 courses of high-dose CHOP (cyclophosphamide 3 g/m^2, doxorubicin 75 mg/m^2, vincristin 2 mg, prednison 300 mg/m^2 + G-CSF every three weeks (21 pts). Protocol 2: 3 courses of standard CHOP-21 followed by 3 courses of ESHAP (26 pts). Protocol 3: 3 courses of high-dose CHOP + 3 courses of ESHAP (29 pts). Protocol 4: same as protocol 3 with addition of rituximab to each cycle of chemo (59 pts). PBPCs were mobilized after 2nd or 3rd high-dose CHOP in protocol 1 and after 1st or 2nd ESHAP in protocols 2,3,4. Patients in complete or partial remission after all types of induction treatment were consolidated with BEAM and ASCT. IF radiotherapy was administered to initial bulk or to residual mass after chemo. CNS involvement at diagnosis was an exclusion criterion. Intrathecal CNS prophylaxis consisted of 15 mg methotrexate +- 40 mg ara-C and it was recommended but not mandatory part of the protocols. Intrathecal prophylaxis received 50% of patients with median number of 3 cycles for patient (range 1-8). *Results.* The median age of the whole cohort was 46 years, male/female ratio 76/59. 58% had IPI 2 and 42% IPI 3. We observed 9 CNS relapses (7%), 7 on therapy, one 1 month after completion of the therapy and one late relapse following 15 month after diagnosis. Original histologies in CNS relapsing patients were DLBCL 6x, 1x mediastinal DLBCL, 1x PTL, 1x FL. 5 of these patients received CNS prophylaxis (all 5 pts received i.t. MTX 15 mg+ara-C 40 mg, median 5 cycles). Median time from study entry to CNS recurrence was 5 month (range 2-15). Median survival after diagnosis of CNS relapse was only 1 month (0,1 -16). No patient after CNS relapse is alive, 8 died due to progression, one after 16 month in next CR due to pneumonia. Evaluated risk factors for CNS progression were IPI, clinical stage, B symptoms, performace status, LDH level, intrathecal prophylaxis and type of treatment protocol. None of these risk factors were significantly predictive for CNS relapse. *Summary/Conclusions.* Incidence of CNS poression/relapse in this cohort of high risk lymphoma patients is relatively low, but outcome of all patiens is fatal. Our patients did not benefit from intrathecal prophylaxis. More precise detection of patient at risk for CNS involvement and detection of occult disease at diagnosis are needed to differentiate the treatment protocols with appropriate CNS prophylaxis for these patiens.

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0701

BURKITT AND NON-BURKITT TYPES OF CHIDHOOD B-CELL LYMPHOMAS (B- NHL) - COMPARISON OF TREATMENT *RESULTS*. A REPORT OF POLISH PAEDIATRIC LEUKEMIA/LYMPHOMA STUDY GROUP (PPLLSG)

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Background. The efficacy of the LMB-89 protocol for children with NHL-B has been investigated. The patients (pts) were treated in 10 oncohematological centers of PPLLSG between 1993 and 2006. A total number of 149 children with NHL-B were included into analysis: 105 (70%) of them with of B-NHL Burkitt (I gr.) and 44 (30%) with B-NHL non ' Burkitt (II gr.) histopathological types (17 - Burkitt-like, 6 Large B-cell, 4 immunoblastic, 6 centroblastic, 11 others). Median observation time was 56 months. *Methods.* The diagnosis was based on histomorphological investigation and supplemented with immunophenotyping. The S. Murphy staging system was used for prognostic stratification. Treatment intensity was adapted to 3 risk groups (A,B,C), according to LMB-89 protocol. *Results.* In both groups majority of children presented on admission advanced stage III and IV disease (67% and 15% for I gr. and 45% and 13% for II gr., respectively). Eighty-two (82%) pts were classified to B risk group. Complete remission (CR) was achieved in 94 (90%) pts with Burkitt and 42 (96%) pts with non-Burkitt types: 16 (94%) - Burkitt-like, 6 (100%) - Large B-cell, 6 (100%) ' centroblastic, 3 (75%) ' immunoblastic, 11 (100%) ' others. There were 13 (9%) non'responders: 11 in I gr. and 2 in II gr. 8 early deaths were observed: 7 in I gr. (4-advanced tumour in diagnosis, 1-acute renal failure+peritonitis,1-St.aureus sepsis+varicella, 1-multiorgan failure+ myelosupression) and 1 in II gr. (advanced tumour in diagnosis). 11 relapses were observed: 7 in I gr. and 4 in II gr. (1- Burkitt like, 1- centroblatic, 2 ' others). 8 pts died after RC: 5 in I gr. due to disease relapse, 3 in II gr. (2 'relapse, 1' toxicity related death (lungs failure)). Probability of EFS was 0.86 for all pts (in previous study 0,73). The EFS of I + II, III and IV stages were: 0,93, 0,87 and 0,71 respectively (p=0,02). The EFS for B- NHL Burkitt was 0.83 (in previous study 0.81) and for non-Burkitt -0.87. *Conclusions.* The treatment outcome of children with B- NHL, esspecially with Burkitt type has improved in comparison to previously reported observations. Higher EFS and overall survival of B-NHL, including Burkitt type, could be achieved thanks to quick diagnosis after first tumour clinical symptoms and an improvement of intensive supportive care (adequate blood product substitutions, regular infection specific prophylaxis, amelioration of MTX therapy monitoring) for therapy toxicity elimination (compared with previous study). The worst results are observed in children with bone marrow involvement and in those with large tumor burden at diagnosis in two examinated pts groups.

0702

HAEMATOLOGICAL AND EXTRAHAEMATOLOGICAL MALIGNANCIES AFTER AUTOLOGOUS TRANSPLANTION FOR LYMPHOPROLIFERATIVE DISEASES

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Background. Secondary myelodysplastic syndrome (sMDS), acute myeloid leukemia (sAML) and solid neoplasia are serious complications of high-dose chemoterapy followed by autologous bone marrow (BMT) or peripheral blood stem cell transplantation (PBSCT) for lymphoproliferative diseases (LPD). These complications are associated with very poor prognosis. *Aims.* We evaluated the incidence of secondary malignancies after autologous PBSCT for non-Hodgkin's lymphoma (NHL), Hodgkin's disease (HD), multiple myeloma (MM) or chronic lymphocytic leukemia (CLL). *Methods.* We studied 142 patients (pts) affected by LPD (79 NHL, 31 MM, 26 HD, 6 CLL) undergone from 1988 to 2004 to autologous transplant. At PBSCT all patients showed absence of karyotypic abnormalities. We considered a minimum of 12 months of follow-up after PBSCT without receiving chemo-radiotherapy in order to assess sMDS without interference of conditioning regimen. The evaluation of sAML and secondary solid neoplasia was done starting from transplant without any delay. The patients withdrew from the study for progression disease (PD) requiring treatment or patient's death. The evaluation, including morphological studies of bone marrow aspiration and peripheral blood's smears, cytogenetic and FISH analysis, was performed after 12 months from PBSCT and at least every 12 months during the follow-up. Eighty-one pts were male, 61 were female with median age of 44 years (range 17-63). At PBSCT 69 pts were in complete remission (CR), 64 pts in partial remission (PR) and 9 pts in PD. Before transplant, after front-line therapy, 53 pts underwent second line chemotherapy, 26 third line or more, 26 pts second line plus rituximab, 7 pts second line plus radiotherapy and 17 pts second line plus first PBSCT. *Results.* Median follow-up was 48 months from PBSCT (range 12-182) for a total of 376 morphologic evaluation, 256 cytogenetic analyses and 46 FISH analysis. We observed one case of sAML with deletion of chromosome 7 occurred 10 months after PBSCT in pt affected by heavily pre-treated NHL (5 lines chemotherapy); he died 2 months later in progression disease and two cases of MDS. The first patient with CLL developed sMDS (refractory cytopenia with multilineage dysplasia) with del (20) detected by FISH and cytogenetic analysis 15 months after PBSCT; he is alive 28 months after sMDS without therapy. The second patient affected by heavily pre-treated LNH (Second line chemotherapy plus rituximab) developed AREB-II with -7 detected by FISH 41 months after PBSCT. Three pts developed secondary solid malignancies (lung, pancreas, colon) at median time of 32 months (range23-126). *Summary/Conclusion.* MSD/sAML are very late complications after allogenic transplant, while recent studies observed an increased incidence after autologous PBSCT for LPD. This evidence suggests that their development is resulted from accumulating mutations in the recipient cells caused by prior exposure to subablative chemotherapy. A stringent and complete follow-up is requested in order to rapidly detect these complications offering to the pts a chance of treatment.

0703

THE COMPARISON OF PERIPHERAL T-CELL LYMPHOMA WITH DIFFUSE LARGE B-CELL LYMPHOMA WHEN TREATED WITH SAME FIRST LINE CHOP CHEMOTHERAPY

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Background. The prognosis of peripheral T-cell lymphoma, unspecified (PTCLu) is usually regarded poor when compared with that of diffuse large B-cell lymphoma (DLBL). Previously published papers, however, gave no consistent data regarding the differences between two lymphomas and enrolled many various cell types as well as different treatments resulting in obscure observation for a certain lymphoma type. *Aims*. We wanted to compare the clinical features and prognosis of T-cell lymphoma with those of aggressive B-cell lymphoma, especially between PTCLu and DLBL when same first-line chemotherapy was applied. *Methods.* Patients with PTCLu and DLBL were selected when their first-line chemotherapies were CHOP. Clinical data were collected through retrospective review of medical records. Progression-free survival (PFS) and overall survival (OS) were calculated from the first day of CHOP chemotherapy.

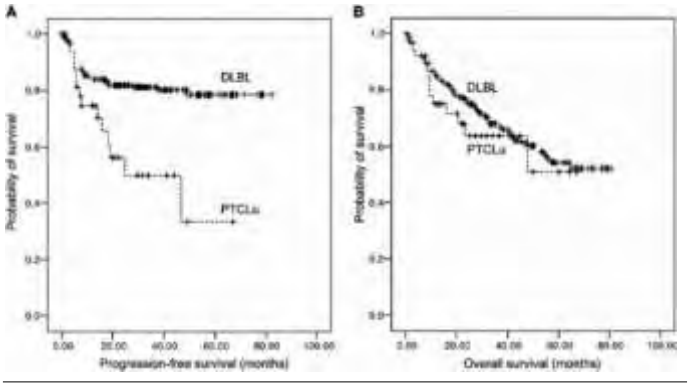

Figure 1. PFS and OS.

Results. From Nov 1997 to Sep 2005, 397 patients received CHOP as first-line chemotherapy. Among them, 290 and 107 patients were classified as B-cell and T/NK-cell lymphoma, respectively. Total 238 patients with DLBL and 40 patients with PTCLu were analyzed in this study. Gender (p=0.530) and age at diagnosis (p=0.750) were not different between patients with PTCLu and DLBL. Patients with PTCLu were more in advanced stage (p=0.003) and had more B symptoms (p=0.007). The number of extranodal involvement (p=0.114), LDH (p=0.816), IPI (p=0.151), overall response rate (89.1% vs. 80%, p=0.544) and time to best response (3.39 vs. 3.06 months, p=0.5201) were not different between two groups. However, complete remission (CR) rate to CHOP was higher in DLBL (75.2% vs. 60%, p=0.045). The progression rate (21% vs. 47.5%, p=0.001) and frequency of second-line chemotherapy (24.7% vs. 40%, p=0.017) were higher in PTCLu. PFS was shorter in PTCLu (median, 24.87 months) than in DLBL (median, not reached; p=0.0004; Figure 1A). On the other hand, CR rate, objective response rate and progression rate to second-line chemotherapy were similar in both group (25% vs. 27.7%, p=0.836; 44.7% vs. 56.3%, p=0.424; 53.8% vs. 50%, p=0.813, respectively). OS of PTCLu and DLBL were similar (medi-

an, both not reached; p=0.4019; Figure 1B). *Summary/Conclusions.* PTCLu showed more frequent relapse rate and poor PFS after CHOP chemotherapy, in spite of similar response rate to fist-line CHOP chemotherapy compared with DLBL. Responses and relapse rate to second-line chemotherapy were similar and OS was not different in both groups.

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0704

RITUXIMAB-CHOP AND RADIOTHERAPY FOR PRIMARY MEDIASTINAL LARGE B-CELL LYMPHOMA: AN UPDATE

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Background. MACOP-B or even chemotherapy (CT) with consolidation high dose therapy with autologous stem cell support (HDT-ASCT) have been considered superior to CHOP in PMLBCL. However, in the absence of randomized trials, there is no established optimal treatment for these patients. Recent data have shown that R-CHOP is superior to CHOP in patients with diffuse LBCL, so that it is rapidly becoming the new standard of care for this subtype of aggressive lymphoma. In younger, intermediate/high-risk patients with aggressive lymphomas HDT-ASCT was superior to conventional CT in the pre-rituximab era, but its role in the era of rituximab is unclear. Thus the role of R-CHOP in the particular case of PMLBCL, which usually affects young patients, is not well established yet. *Aims.* The evaluation of the efficacy of R-CHOP±RT in PMLBCL and the comparison of this approach with CHOP±RT, administered to historical controls. Patients and *Methods.* Between 1994 κα 2005, 62 patients with PMLBCL were treated in 4 participating centers. R-CHOP displaced CHOP in the treatment of PMLBCL at a given timepoint in each center. Thus 23 consecutive patients who received R-CHOP, were compared to 39 consecutive historical controls, who had been treated with CHOP prior to that point. *Results.* The median age of the patients was 32 years (17-63) and 42/62 (68%) were females. Age-adjusted IPI was ≥2 in 39% and 46% of patients who received R-CHOP and CHOP respectively (p=0.61). All individual IPI parameters as well as B-symptoms were also balanced between the two groups. The complete response (CR) rate was 100% for R-CHOP±RT vs 64% for CHOP±RT (p=0.001). All relapses after CHOP occurred within 22 months from diagnosis. No relapse has been recorded after R-CHOP, while a single patient with CR but persistent PET abnormality underwent stem cell transplantation and was considered as failure .The 3-year failure free survival (FFS) was 95±4% vs 51±8% for patients who received R-CHOP±RT vs CHOP±RT (p=0.001). Within the subgroup of patients with L/LI risk IPI the corresponding 3-year FFS rates were 100% vs 57±11% (p=0.008), while they were 89±10% vs 44±12% (p=0.04) among patients with HI/H risk IPI. The 3-year event free survival (EFS) for all patients was 91±6% vs 51±8% (p=0.003). The 4-year overall survival was 96±4% vs 66±8% (p=0.03), while the 4-year lymphoma specific survival was 100% vs 66±8% (p=0.008). *Conclusions.* R-CHOP and RT provided impressive results with only one failure and lymphoma-related deaths among 23 patients. In comparison to CHOP-treated historical controls, highly significant differences in favor of R-CHOP were recorded in terms of CR, FFS, EFS, and LSS rates. Overall survival was also improved. Based on these results we continue to treat PMLBCL patients with R-CHOP and RT. The need for more aggressive strategies, such as MACOP-B or ASCT, is therefore questionable. Whether RT is needed after R-CHOP, especially when post-chemotherapy PET-scan is available, should be investigated.

0705

TREATMENT STRATIFICATION ACCORDING TO EARLY RESPONSE TO MEGA-CHOP, BASED ON CT AND GALLIUM 67 SCAN WITH OR WITHOUT IFE SALVAGE THERAPY FOLLOWED BY AUTOLOGOUS STEM CELL TRANSPLANT IN PATIENT WITH POOR PROGNOSIS AGGRESSIVE LYMPHOMA

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Background. Patients with IPI ,23 large cell lymphoma have a poor outcome with long term survival lower than 50%. Evaluation of response only with CT scan shows often residual masses which can be tumoral or fibrotic. Gallium 67S discriminate better these two situations and therefore can help to decide further strategies. *Aim.* To assess the efficacy of PBSCT in patients with poor prognosis aggressive NHL according to previous early response to Mega-CHOP evaluated with CT & Ga67S. *Patients and Methods.* Inclusion criteria were: G67S positive large cell B cell lymphoma with IPI score > 2 or IPI <2 with high β2 microglobulin or peripheral T cell lymphoma (PTCL), except ALK+ anaplastic lymphoma regardless of IPI. Patients were evaluated after 3 cycles of Mega-CHOP. Those in CR (CT scan, Ga67S negative) or uCR (CT scan positive, Ga67S negative) received a 4rd Mega-CHOP followed by BEAM and PBSCT. Those with positive Ga67S received IFE or ESHAP (x2) regimens followed by BEAM and PBSCT. Patients with refractory disease (RD) were dropped from the study. Since 2001, 112 patients have been registered and 87 have finished the treatment. Median age was 52 years (20-67 years) and 49% were males. Seventy one (72%) had a DBLCL, 8 (7%) a grade 3 FL and, 24 (21%) PTCL. Sixty two (88%) had IPI > 2, and 12% IPI 1. Doses were for Mega- CHOP : Cy 1,5 g/m^2, ADR 65 mg /m^2 and VCR 2 mg on day 1 and Pred 60 mg /m^2 days 1 ‘ 5) on a 21 day schedule and for IFE : Ifosfamide 10 gr/m^2 and VP16 900 mg/m^2 (days 1-3) with Mesna. *Results.* After 3 Mega-CHOP, 47 patients (42%) were considered on CR or uCR due to a negative Ga67S, 46(41%) were on PR and 18 (16%) were refractory. One patients were early deaths. After IFE 18/46 (39%) achieved CR, 19 (41%) PR and 9 (20%) progressed. Overall, 87 patients received PBSCT and are valuable for response. Thirty one patients (28%) died, 23 (21%) due to lymphoma and 8 (7%) due to toxicity. With 36 months of median follow-up (8 to 69 months), 81 patients are alive, 67 (60%), disease free. Five-year overall survival according to clinical response and Ga67S after the 3 initial Mega-CHOP were 70% (CR, PR and Ga67S neg.), 67% (CR. PR Ga67S pos.) and 37% (failure Ga67S pos.), respectively (p=0.0004). In the univariate analysis, the significant variables associated with outcome were clinical response combine with positive/negative Ga67S after the initial therapy (p=0.0004) and disease status at ASCT (p=0.01). *Conclusion.* Our preliminary results suggest that early salvage therapy can overcome the poor outcome of patients with bad prognosis aggressive lymphoma. Moreover, this early evaluation could identify patients with poor prognosis who only need a short treatment (4 Mega-CHOP+PBSCT).

0706

PRELIMINARY RESULTS FROM A PHASE II STUDY OF LENALIDOMIDE MONOTHERAPY IN RELAPSED/REFRACTORY AGGRESSIVE NON-HODGKINS LYMPHOMA

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Background. Lenalidomide (Revlimid®) is an immunomodulatory drug of the IMiD class, recently approved in the US for myelodysplastic syndromes associated with a deletion 5q[31] cytogenetic abnormality that also has activity in multiple myeloma, chronic lymphocytic leukemia and cutaneous T-cell lymphoma. Thalidomide, a less potent IMiD, has activity in non-Hodgkin's lymphoma as both monotherapy and in combination with rituximab. *Aim.* To assess the safety and efficacy of lenalidomide monotherapy in subjects with relapsed/refractory aggressive non-Hodgkin's lymphoma (NHL). *Methods.* Subjects with relapsed/refractory aggressive NHL following > 1 prior treatment regimen with measurable disease are eligible. Subjects receive 25 mg lenalidomide orally once daily on Days 1-21 every 28 days and continue therapy for 52 weeks as tolerated or until disease progression. Response and progression are evaluated using the IWLRC methodology. *Results.* 19 subjects of a planned 40 were enrolled of which eight subjects are currently evaluable for tumor response and safety. The median age of the 8 evaluable subjects is 66 (45-80) and 5 are female. Histology is diffuse large cell lymphoma (n=7) and follicular center lymphoma grade 3 (n=1). Median time from diagnosis to lenalidomide monotherapy is 2.3 (1-6) years and median number of prior treatment regimens per subject is 3 (1-6). Median duration of follow-up is 3.5 (1-5) months. Three of the eight subjects exhibited a PR with decreases in their tumor burden of 93%, 79% and 52%. Two subjects had stable disease and three, disease progression. Grade 3 or 4 hematological adverse events (neutropenia, thrombocytopenia, anemia) occurred in five subjects including one febrile neutropenia and one of the five also exhibited Grade 3 sub-acute autoimmune hemolysis and Grade 4 general malaise. *Conclusion.* Preliminary data for lenalidomide monotherapy in relapsed/refractory aggressive NHL are encouraging.

0707

RITUXIMAB SIGNIFICANLY IMPROVES THE OUTCOME OF YOUNG POOR RISK PATIENTS WITH DIFFUSE LARGE B-CELL LYMPHOMA - ON BEHALF OF CZECH LYMPHOMA STUDY GROUP

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Background. There is a robust evidence of significant patients outcome improvement by adding rituximab (R) to chemotherapy (CHT) in patients (pts) with DLBCL who are older (Coiffier, 2002) or younger with good risk profile (Pfreundschuh, 2004). There is lack of evidence of benefit R-CHT over CHT for younger pts with DLBCL and poor risk profile according to IPI and moreover the benefit of combination of rituximab and primary high dose therapy (HDT) with autologous stem cell transplantation (ASCT) is unclear. *Aim.*To perform the retrospective analysis of pts with DLBCL and intermedate-high (IH) or high (H) aaIPI, younger than 60y registered in CLSG registry since Jan 1999 till Dec 2004 and treated with anthracyclin containing chemotherapy and to compare the chemotherapy only treated group (CHT) vs rituximab and CHT (R-CHT) treated group. *Methods.* Altogether 178 eligible pts were identified, 118 (66.3%) with CHT nad 60 (33.7) with R-CHT. The median of rituximab infusions was 6 (4-8) and 5 pts with less than 4 cycles of R were counted as CHT only pts. There were no significance difference in CHT vs R-CHT in terms of age (median 47 in both), clinical stage (advanced 92.4% vs 95%), elevated LDH (91.5% vs 89.8%), H risk aaIPI (42.4% vs 35%), radiotherapy as part of the induction (41% vs 54.7%). The only difference between groups was in the number of pts exposed to HDT with ASCT (38.5% vs 60%, p=0.01). The median follow up in CHT group was 4.6 years vs 2.4 in R-CHT group. The 3 years probability of overall survival - OS - and event free survival *EFS* (time from dg to progression/relaps or death, whatever occured earlier, in all pts) were considered as primary endpoints. Epiinfo and GraphPad programs were used for analysis (ANOVA, Wilcoxon test and log rang tests were used). *Results.* The probability of EFS and OS in the whole group was 52% and 61% resp. The probability of EFS in CHT vs R-CHT was 40.1% vs 74.8% (p<0.0001) resp. and OS was 50.8% vs 83.2% (p<0.0001). Because of inbalance in the HDT with ASCT, the subanalyses were performed. The comparison of subgroup of pts who all were treated with HDT as part of the induction according to R administration (CHT vs R-CHT) reveals the significant differences for EFS: 55.5% vs 88.8% (p<0.005) as well as for OS: 61.4 vs 91.4 (p<0.01). There were also singinficant differences between CHT vs R-CHT groups when pts without primary HDT were analyzed: EFS: 32.9% vs 50.9% (p<0.02) and OS: 45.0% vs 67.7% (p<0.01). There was found no difference between intermediate-high and high subgroups. *Conclusion.* This retrospective analysis suggests: Young pts with DLBCL and poor risk IPI have significantly better outcome if they are treated with rituximab containing chemotherapy. Moreover the R-CHT significantly improves the outcome of patients who are designated to HDT with ASCT in comparison of pts who are treated with CHT without R followed by HDT with ASCT.

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0708

WHATS THE SIGNIFICANCE OF FDG-PET/CT SCAN AT DIAGNOSIS OF NON HODGKIN LYMPHOMAS?

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Background. Correct staging is important for the appropriate treatment in lymphoma patients. Most cancers, including lymphomas, metabolize glucose at abnormally high rate and so FDG-PET/CT is an important tool in the evaluation of patients with lymphoma. Many authors in these last years have shown the importance of FDG-PET/CT analysis at diagnosis of lymphomas and the differences according to histologic subtypes. *Aims.* The IIL (Italian Lymphoma Intergroup) evaluated:1) the role of FDG-PET/CT versus CT scanning in the staging of Non-Hodgkin's lymphoma, 2) the significance of FDG-PET/CT according to histologic subtypes, 3) the ability of FDG-PET/CT in showing extranodal localizations. *Methods.* We have retrospectively analysed at diagnosis 105 patients (pts) (53 male, 52 female) with both FDG-PET/CT and conventional CT scanning. The histologic subtypes were: diffuse, large B-cell lymphoma (LBCL) 49 pts (47%), follicular lymphoma (FL) 37 pts (35%), marginal zone lymphoma (MZL) 7 pts (6%), mantle cell lymphoma (MCL) 4 pts (4%), Burkitt and Burkitt-like lymphoma (BL) 3 pts (3%), primitive mediastinal B-cell lymphoma 2 pts (2%), other lymphomas (small lymphocytic, peripheral T-cell, extranodal) 3 pts (3%). The PET/CT scans (GE, Discovery, LS) were performed 60 min. after the i.v. injection of 18F-FDG (5.5 MBq/kg) with a whole-body acquisition with a field of view extending from the head to the upper part of the thighs. All patients fasted for at least 8 hours prior to FDG injection and had a glucose level < 120 mg/dL. *Results.* We have evaluated nodal (18) and extranodal (12) stations. Considering all cases, the agreement between FDG-PET/CT and CT scanning was 89% in nodal stations and 95% in extranodal ones, while discordance was 9% (7% toward PET/CT and 2% toward CT), and 5% (4% toward PET/CT and 1% toward CT) respectively. The percentage was similar in all the different histologic subtypes. The extranodal localizations in which there were more discordances were spleen (7 pts), liver (6 pts), and bones (17 pts. FDG-PET/CT upstaged 27/105 pts (26%) and for 17% of pts the upstaging modified therapy (0 → III-IV in 4 pts (4%), I→ III-IV in 3 pts (3%), II → III-IV in 10 pts (10%). The FDG-PET/CT downstaged only 9/105 pts (9%): II→ I in 1 pts (1%), III-IV → II in 5 pts (5%), I → 0 3 pts (3%). *Conclusions.* FDG-PET/CT and CT scanning are concordant, for nodal and extranodal localizations, in staging of Non-Hodgkin lymphomas. FDG-PET/CT shows more nodal localizations (7%) and extranodal localizations (4%) than CT scanning. There isn't s substantial difference in concordance between FDG-PET/CT and CT scanning according to the various histologic subtypes. It is important to have FDG-PET/CT baseline for early and late evaluation during and after therapy. FDG-PET/CT is essential for staging lymphomas also as exclusive method.

0709

EXTRA-NODAL NON-HODGKIN LYMPHOMAS AT A COMPREHENSIVE CANCER CENTRE IN PORTUGAL

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Background. Non-Hodgkin Lymphomas (NHL) can arise within any organ in the human body. Extra-nodal NHL account for about 25% of the total cases of lymphoma diagnosis. *Aim.* To evaluate the relative frequency of adult extra-nodal NHL and their absolute and relative outcome in the last five years in a Portuguese comprehensive cancer centre. *Methods.* Retrospective analysis of a cohort of adult patients with histologicaly confirmed diagnosis of extra-nodal NHL between January 2001 and December 2005 treated at Instituto Português de Oncologia ' Porto. A lymphoma was classified as extra-nodal when the biggest lymphomatous mass was located outside a lymph node and patient's chief complaints were attributable to it. Patients with Burkitt's lymphoma and lymphoblastic lymphoma were excluded. Data on demographics, histology, stage according to Ann Arbor system, known prognostic factors and treatments performed were collected. Descriptive analysis was performed as appropriate for each variable. Overall survival and survival as per anatomical location were analysed. Significance of prognostic factors on survival were evaluated by Log Rank test. *Results.* A total of 119 patients were identified. The mean age at diagnosis was 58,4 years (range 19-86). Fifty two percent of patients were male. The most prevalent locations were the stomach with 54 cases and the skin with 15 cases. All other locations were represented with less than 10 cases each. Treatment differed according to the extra-nodal location and to the specific patient and disease characteristics. Median time of follow up was 20 months. The median survival was not reached either for the whole population or for any of the more prevalent locations. Survival at 24 months was 70%. Overall 32 deaths were registered. In univariate analysis, survival was significantly worse for age above 60 years (n=119, p=0.01), ECOG of 2 or more (n=117, p<0.0001), LDH above normal (n=109, p<0.0001), albumin below normal (n=110, p=0.0001) and Ann Arbor stage III or IV (n=116, p<0.0001). *Summary/Conclusions.* In this single institution series, as with most reported series, the most prevalent extra-nodal location for NHL was the stomach. Survival at two years of follow up was 70%. Median survival was not reached due to short follow up. Prognostic factors for nodal lymphoma appeared to be valid when applied to this subgroup of lymphoma patients.

Non-Hodgkin's Lymphoma - Clinical IV

0710

INTERLEUKIN-8 AND INTERLEUKIN-10 LEVELS IN DIFFUSE LARGE B-CELL LYMPHOMA: CORRELATION WITH INTERNATIONAL PROGNOSTIC INDEX AND OUTCOME

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Background. Cytokines play important roles in the pathogenesis of lymphomas. The secretion of cytokines can provide growth advantages for tumor cells in either an autocrine or a paracrine fashion. An elevated serum or tissue level of cytokines can contribute to the clinical and histopathologic alterations associated with malignant lymphomas. The aim of this study was to determine the relations between serum levels of interleukin-8 (IL-8) and interleukin-10 (IL-10) and outcome in diffuse large B-cell lymphoma (DLBCL). *Methods.* Serum levels of IL-8 and IL-10 were measured using a sensitive enzyme-linked immunosorbent assay in the pretreatment frozen serum from 46 patients with diffuse large B-cell lymphoma and from 30 healthy control subjects. In 43 cases initial treatment consisted of CHOP and three of ACVBP regimen. The median follow-up duration was 49 months (33-68months). Cytokine levels were correlated with clinical features and survival. *Results.* Serum IL-8 levels were higher in NHL patients (median undetectable; range: undetectable to126 pg/mL) than in control subjects (median undetectable; range: undetectable to 28.9 pg/mL) (p=0.001). Serum IL-10 levels were higher in NHL patients (median 4.9 pg/mL; range: undetectable to 299.2 pg/ml) than in control (median undetectable; range: undetectable to 22.2 pg/ml) (p<0.001). In 34.8% of patients two cytokines were elevated in parallel. Patients with DLBCL were divided in high- and low-risk group according to International Prognostic Index (IPI). In the high-risk group, serum levels of IL-8 (range: undetectable-126; median 20.8 pg/mL) were also higher than serum levels of IL-8 (range: undetectable-101.9; median undetectable) in the low-risk group (p=0.015). In addition, IL-10 in high-risk group were found to be higher (range: undetectable-299.2; median 30.7 pg/mL) than serum levels of IL-10 in the low-risk group (range: undetectable-56.6; median undetectable) (p<0.001). Superior response to therapy (complete remission) was achieved in patients with lower serum levels of IL-8 (p=0.011) and IL-10 (p<0.001). Patients who were effectively treated had a significant reduction in cytokine levels (p<0.05). Patients with elevation of serum IL-10 had poor median and 5-year survival (p=0.007), while IL-8 levels did not affect overall survival (OS). Using univariate analysis, overall survival in all patients was affected by presence of systemic symptoms, Ann Arbor stage, performance status score, elevated levels of β-2 microglobulin, pretreatment serum levels of cytokines IL-8 and IL-10 and the number of cytokines increased. *Conclusions.* Serum assay of IL-8 and IL-10 before the treatment in patients with newly diagnosed DLBCL may help us to have some perception about the possible prognosis and to decide on therapeutic approaches for individual patients.

0711

ANTI-LEUKEMIC AND ANTI-ANGIOGENESIS EFFICACY OF ARSENIC TRIOXIDE IN NEW CASES OF ACUTE PROMYELOCYTIC LEUKEMIA

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Arsenic trioxide is now considered the standard agent in treatment of refractory cases of acute promyelocytic leukemia (APL). This drug is also shown to have anti-angiogenesis effect against APL cells *in vitro*. This study evaluated clinical efficacy and anti-angiogenesis effect of arsenic trioxide in 17 new cases of APL. Arsenic trioxide was given in a dosage of 0.15 mg kg^{-1} and remission rate, survival rate, toxicities and effect on vascular density of bone marrow was studied. The bone marrow vascular density was examined using immunohistochemistry for von Willebrand Factor (vWF) and CD31 markers. Bone marrow vascular density was determined by calculating mean vessel number in 3 hot spot, high power microscopic fields. Bone marrow vascular density was reduced as identified by anti-vWF immunohistochemical staining (Mean before treatment = 201.6 mm^{-2} ± 20.4 (SEM), mean after treatment = 109.4 ± 17.2 (SEM), p< 0.001) and anti-CD31 immunostaining (mean before treatment = 199.17 mm^{-2} ± 21.5 (SEM), mean after treatment = 99.5 mm^{-2} ± 22.1 (SEM), p<0.05). Treatment efficacy results showed

100% complete remission rate after median of 30 days and 72% survival rate after median 860 days of follow-up. Main toxicities included hyperleukocytosis, hepatic toxicity and APL differentiation syndrome. The results imply that arsenic trioxide is an effective anti-leukemia and anti-angiogenesis agent in new cases of APL.

0712

SALVAGE TREATMENT WITH HIGH-DOSE THERAPY AND PSCT IN HIGH GRADE NHL - FROM THE GISL (GRUPPO ITALIANO STUDIO LINFOMI)

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Background. The inclusion of high-dose therapy and PBSCT as salvage treatment in patients with HG-NHL is generally planned in designed protocols. However, it is generally difficult to apply the therapy to all expected patients due to several reasons, including the disease progression which leads frequently to change the assigned therapy, or to compliance of patients with high-dose therapy, or to difficult to obtain an adequate number of CD34 cells. *Aims.* We focus the study on a population of young patients with HG-NHL addressed to salvage treatments including high-dose therapy. The analysis is finalized to show if there are difference on survival according to an intention to treat (ITT) between those addressed to a conventional (CT) or to an high-dose (HDS) salvage treatment, either in patients relapsed or in those primarily resistant to previous therapy. *Methods.* Since 1988 up to 2003, 1122 patients with HG-NHL completed the assigned therapy according the undergoing protocols; 236 patients of age < 60 years were assigned to a salvage program either because primarily resistant (103=44%) or relapsed (133=56%). Based on ITT analysis we valuated CT or high-dose therapy HDS in 181 valuable patients. One hundred twenty one patients were assigned to CT (67%) and 60 to HDS (33%). Among the 60 patients addressed to HDS 38 (63%) did the planned therapy and 22 didn't, 62% were refractory and 65% relapsed. Crude results show in refractory patients 14% CR with CT and 30% with HDS; instead relapsed patients obtained 45% and 48% CR with CT and HDS, respectively. Relapse following salvage therapy, occurred in 70% and 36% of refractory patients treated by CT and HDS, respectively, and in 40% and 27% of relapsed patients treated by CT and HDS, respectively. The OS of 80 refractory patients according to ITT analysis show 8 months median survival, 6 and 10 months for CT and HDS, respectively; the 3 years survival is 22% and 32%, respectively (p=0.142). The analysis done for the therapy effectively performed show in refractory patients a median survival of 6 and 18 months for CT and HDS, respectively (p=0.056). The OS of relapsed patients is 26 months median, 21 and > 84 months for CT and HDS, respectively. The 3 years survival is 38 and 70%, for CT and HDS, respectively (p=0.029). The analysis for therapy effectively done show a better significance of survival according to two arms of treatment (p=0.025). *Conclusions.* Our study demonstrated that HDS in relapsed patients with HG-NHL exerts a better outcome than CT, whether the same result could be obtained in refractory patients should be further investigated by prospective studies even if a positive trend could be already disclosed in this subset of patients.

0713

THE EFFICACY OF RITUXIMAB PLUS IFOSFAMIDE SECOND LINE APPROACH IN RELAPSED/ REFRACTORY NON HODGKIN LYMPHOMA

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Background. relapsed high grade NHL is a very aggressive pathology characterised by a worse prognosis. High dose therapy followed by peripheral blood stem cell (PBSC) rescue, is to date the gold standard therapy: patients who undergo transplant in complete response have better progression free survival with respect to those in partial response. The addition of Rituximab proved to increase CR rate before transplant. *Aims.* to test the efficacy of combined immuno-chemotherapy in 2nd line treatment we designed a protocol including Rituximab+ IEV. *Methods.* the planned treatment consists of 3 cycles of IEV or MINE (>65 y patients), plus Rituximab (375 mg/sqm) given before (day 1) and after (day 14) every cycle (six doses). G-CSF was administered from day 7 to haematological recovery and, after the third cycle, was continued until the end of apheresis. From April 2002 to December 2005 23 consecutive patients - 16 males, 7 females, median age 57 y (min. 21 max. 73 y) with refractory (9) and relapsed (14) Diffuse Large B Cell Lymphoma (DLBCL) entered in the study. According to IPI score 15 patients had intermediate- high grade NHL, 10 had extranodal disease, finally 4 were hepatitis C virus positive. *Results.* nineteen patients-17 R-IEV and 2 R-MINE-completed treatment and are evaluable, the remaining 4 are not evaluable because of discontinuation treatment (2), lost to follow up (1) and death (1). Of 19 evaluable, 11were responders: 6 achieved CR, 5 PR, while 6 in progressive disease (PD) went off-study. Toxicity was mainly haematological: > 3 WHO grade neutropenia, anemia and thrombocytopenia were recorded in 17, 7 and 12 pts, respectively. CD 34 harvest was successful in 11 pts: CD 34+ median value 7,6 x 10 6 /Kg. Among the responders 1 went off study because of no CD 34 harvesting and 2 for disease progression. Eight patients underwent transplant, 4 autologous and 4 (>60 y) reduced intensity allogeneic SCT. Of transplanted patients 4 (2 in CR post Mini Allo, 2 in progression post Auto) died 2, 6, 6 and 10 months after transplant, respectively. The remaining 4 (2 mini-allo and 2 auto) are alive in CR which lasts 4+, 32+, 36+, 42+ months. As January 2006, 11 patients are alive: 6 CR, 4 PR, 1 PD, respectively. Overall median survival from diagnosis was 17 months (range 7-49). *Conclusions.* our experience, if limited to a small series of patients, shows that the addition of Rituximab to second line chemotherapy: a) increases response rate even in adverse IPI score cases, b) allows to obtain a good CD 34 harvest and c) represents an effective *in vivo* purging agent.

0714

SPLENIC MARGINAL ZONE LYMPHOMA: A CLINICOPATHOLOGICAL STUDY IN 16 PATIENTS

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Background. Splenic marginal zone lymphoma (SMZL), recently characterized in the WHO classification of lymphoid tumors, is a rare disorder comprising less than 1% of lymphoid neoplasms. Thus, it is not easy to collect a big group of patients, and only few series have been published. *Aims.* To analyse SMZL cases from four hospitals in our community, from a clinical, biological, and pathological point of view, and to compare our findings with those reported in bibliography. *Methods.* We retrospectively studied 16 cases of SMZL, who were diagnosed in our community over the last eight years (mean age 59, range 44-75; 11 males, 5 females). Analysed data included clinical features at presentation and evolution; analitical, morphological and immunophenotyping findings; presence of chromosomal abnormalities; frequence of extranodal and bone marrow (BM) involvement; infiltration pattern on marrow biopsy; transformation to aggressive lymphoma; response to chemotherapy, and survival rate. *Results.* Patients more frequently presented with splenomegaly (94%), BM involvement (87,5%), BM reticulin myelofibrosis (75%), peripheral blood involvement (62,5%), elevated β2-microglobulin (61,5%), abdominal discomfort secondary to splenomegaly (56%), anaemia + thrombocytopenia (50%) and 'villous' lymphoid morphology (50%). Less frequent were the existence of chromosomal aberrations (37,5%), B symptoms (31%), elevated lactate dehydrogenase (LDH) (25%), hepatomegaly (19%), pancytopenia (19%), positive DAT (18%), and peripheral lymphadenopathies (13%). Lymphomatous-cell immunophenotyping showed positivity to CD20 (100% of analysed cases), CD22 (91%), CD79b (75%), FMC7 (73%), CD5 (45%), and CD25 (40%); only two cases were CD23+ or CD11c+, one expressed CD10+, and none was CD103-positive. Nodular pattern was the most frequent pattern of marrow invasion (6/14 patients), while intrasinusoidal BM infiltration was rarely found (1/14). Extranodal involvement was seen in two patients (12,5%), as specific pleural effusion and ascites. Only one patient (6%) presented with a serum M component (IgA). A chromosome abnormality was detected in three patients, two of them chromosome 7-related. Transformation to aggressive lymphoma occured in three cases. Finally, 4 patients have died, and mean survival is 44 months (range 16-93). *Conclusions.* We did not find significant differences between this serie and those reported in bibliography. Nevertheless, our

patients rarely showed an intrasinusoidal BM infiltration pattern or a serum M component, both reported features of this type of lymphoma. As mentioned by other authors, CD5-positive SMZL cases seem to be more common that previously thought.

0715

IS DOUBLE-BALLOON ENDOSCOPY USEFUL AND NECESSARY FOR THE EVALUATION OF SMALL INTESTINE INVASION IN PATIENTS WITH NHL?

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Background and Aims. It is difficult to diagnose correctly the invasion of non-Hodgkin lymphoma (NHL) in small intestine. Recently, double-balloon endoscopy (DBE) (Fujinon Co. Ltd., Tokyo) has been produced and spread as a new and easy-to-use method of endoscopy for whole small intestine. In this study, we studied the usefulness of DBE in patients with NHL for diagnosis of invasion in small intestine. *Patients and Methods*. From February 2005 until January 2006, DBE was underwent in twelve patients with NHL. Six patients were systemic NHL, five patients were gastric NHL, and one patient was rectal NHL. They were seven males and five females, with an average age of 63.6 years (range: 48 to 78). The pathological findings were 7 diffuse large B cell lymphoma (DLBCL), 2 follicular cell lymphoma (FCL), 1 Maltoma, 1 mantle cell lymphoma, and 1 IPSID. DBE was basically twice done in every case both from mouth and anus on different day as possible. All patients were also underwent biopsy at DBE. *Results.* DBE was safely underwent in all 12 patients. Characteristic endoscopic findings of small intestine were revealed in six patients with NHL. However, in only 4 patients, biopsy specimen showed positive. In the rest two patients, there was no pathological finding of NHL, which was considered due to chemotherapy at the previous hospitals. Both of two FCL cases had endoscopic and pathological findings in the small intestine. They were diagnosed intestinal perforation because of chemotherapy at the previous hospitals. If they were noticed that their NHL was invasive in small intestine, we were able to speculate their small intestine might be performed after chemotherapy. Only 2 gastrointestinal NHL patients had small intestinal lesion. On the other hand, 3 systemic NHL patients had also invasion in small intestine. Especially, IPSID patient was diagnosed with only DBE. Aspiration pneumonia was happened in one patient. Other severe complication was not found. *Conclusions.* DBE was valuable for diagnosis of NHL invasion in small intestine. DBE must be selected before chemotherapy for NHL.

0716

NODAL VS. PRIMARY EXTRANODAL DIFFUSE LARGE B-CELL LYMPHOMAS: A COMPARISON OF PRESENTING FEATURES, RESPONSE TO TREATMENT AND OUTCOME

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Background. Diffuse large B-cell lymphomas (DLBCL) represent the commonest subtype of non-Hodgkin's lymphomas (NHL) in Western countries, comprising 30% of the total. Marked heterogeneity in aspects of morphology, immunophenotype and genetics is their main characteristic. Approximately 30% of them are of primary extranodal origin. It has already been proposed that nodal and extranodal DLBCL could be regarded as two distinct clinical entities, since molecular differences between them suggest a different genetic origin. *Aim.* To assess the main clinical presenting features, response to treatment and outcome of a large number of patients with DLBCL according to the primary site of origin, nodal or extranodal. *Methods.* Between 1976 and 2005, 398 consecutive patients with DLBCL were treated in our department. CHOP and CHOP-like regimens were administered to a total of 353 (88.8%) patients, 60 (17%) of which received additionally radiotherapy, 74 (21%) rituximab and 35(9.9%) both radiotherapy and rituximab. Patients were divided in group A, that comprised 188 (47.2%) patients with DLBCL of primary nodal origin and group B, that consisted of 210 (52.8%) patients with DLBCL of primary extranodal origin. Patients' characteristics (gender, age, stage, IPI, presence of B symptoms, bulky disease and bone marrow infiltration), the kind of treatment (chemotherapy±rituximab±radiotherapy) and response rates were compared between the two groups using $\chi 2$ tests. Disease-free survival (DFS), overall survival (OS) and failure-free survival (FFS) were estimated according to the Kaplan-Meier method. Differences in survival rates were assessed using the log-rank test. *Results.* Group B patients presented with early stage disease (I-II, no bulky disease), low IPI (0-1), no B symptoms, and no bone marrow infiltration with a significantly higher frequency than group A patients (83.8% vs. 45.7%, 62.8% vs. 39.9%, 20% vs. 34.6%, 2.4% vs. 12.2% respectively, $p<0.003$). Patient distribution according to the kind of treatment administered, was not different between the two groups ($p> 0.05$). Median follow-up time for groups A and B was 55 (1-425) and 56 (1-428) months respectively ($p> 0.05$). On an intention-to-treat basis, complete response rates were similar between groups A and B (81.9% vs. 84.8% respectively, $p>0.05$). Actuarial 5-year DFS rate was significantly higher in group B compared to group A (80% vs. 68.3% respectively, $p=0.006$). Actuarial 5-year OS and FFS rates were not significantly different between groups A and B (71.3% vs. 70.3% and 55.2% vs. 61.4% respectively, $p>0.05$). *Conclusion.* In our study, patients with DLBCL of primary extranodal origin demonstrated more favorable presenting clinical features and a higher DFS rate than patients with nodal DLBCL. Nevertheless, OS and FFS rates did not seem to be affected by the primary site of origin.

0717

THE LATE CARDIOTOXICITY OF DOXORUBICIN CONTAINING REGIMENS IN THE TREATMENT OF MALIGNANT LYMPHOMAS

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Backround. Chronic cardiotoxicity of doxorubicin occurs later than one year after completion of the chemotherapy and it represents a serious late treatment related complication. *Aims.* to determine the occurence of late clinical and subclinical doxorubicin cardiotoxicity and the cardiopulmonary performace status in the patients surviving more than 5 year after primary treatment for lymphoma. *Methods.* 96 patients with Hodgkin´s and non-hodgkin´s lymphoma treated in the period 1995 ' 2000 at our department were consecutively included in the prospective study. Male/female ratio 47/49, median age 41 (23-79), median follow-up 6 years (5-10). The maximum cumulative dose of doxorubicin (CD DOX) used in the treatment protocols was 377+147 (median 300, 50-880) mg/m^2. 32 (33%) of the patients received another treatment after primary regimen for high risk disease at the time of diagnosis or for later relapse. Patients were examined by rest echocardiography before initial treatment, after its completion and at the minimum of 5 years folow-up in the survivors. Dynamic stress echocardiography and cardiopulmonary exercise test were performed during control examination. Decline of left ventricular ejection fraction (LVEF) below 50%, progression of decline of LVEF > 10% compared to baseline value and drop-off of peak oxygen consumption pVO2<20 mL/kg/min were considered as pathological. Doppler parameters of left ventricular diastolic function and index of global left ventricular function (myocardial performance index, MPI) were evaluated too. *Results.* Clinical signs of cardiotoxicity were observed in 4% of pts, subclinical cardiotoxocity in 31%. Impairment of diastolic function was present in 38% pts and a pathologic value of MPI in 31% pts. A stress increment of EF was 13+4% (median 12; 5-25). Decreased value of pVO2 was find out in 15% patients. Decrease of LVEF significantly correlates with duration of folow-up after treatment. The risk factors for late cardiac toxicity detected in multivariante analysis were CD DOX > 300 mg/m^2, pre-existence of cardiovascular diseases and age >60 (for CD DOX $p<0.05$; age $p<0.01$; concomitant cardiovascular disease $p< 0.01$, r= 0.57 and $p<0.02$ for whole model). Additional treatment following the initial treatment is associated with higher risk only for finding of diastolic dysfunction (OR=2.37, $p<0.05$), but not for drop of LVEF. Reduced cardiopulmonary performance was diagnosed only in 15% of survivors and is signifficantly affected by age and diastolic impairment. *Summary/Conclusions.* Our data demonstrate, that cardiac function should be long-term monitored at least by means of rest echocardiography in patients after antracycline containing regimens.

0718

PROGNOSTIC SIGNIFICANCE OF GAMMA-DELTA T CELL RECEPTOR EXPRESSION IN BONE MARROW LYMPHOCYTE POPULATION OF LYMPHOMA MALIGNUM PATIENTS

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Background. Gamma-delta T lymphocytes ($\gamma\delta$T) appear to posses intrinsic cytolytic antitumour activity in different carcinomas, sarcomas, myeloma and leukemia. Activated $\gamma\delta$ T cells express antigens CD25+

(late activator) and CD69+ (early activator) on their surface. *Aim.*1) determine a mean percentage (%) of γδ T cells in bone marrow of untreated NHL patients,2) γδ T cells% comparison in bone marrow and peripheral blood of NHL pts,3) verify the impact of γδ T cells presence in bone marrow on the NHL clinical outcomes. *Material and Methods.* 18 newly patients (pts) with NHL diagnosis, admitted to Department of Hematology, Blood Neoplasms and Bone Marrow Transplantation of Wroclaw Medical University between 2002-2005 were included into analysis. The S. Murphy staging system was used for prognostic stratification (pts in III or IV stages). Samples of bone marrow and blood were taken at the time of diagnosis. γδ T cells were estimated by flow-cytometry (FACS), using a fluorescence-activated cell sorter and monoclonal antibodies (MoAbs: Ab-anti TCRγ1-FITC (Becton-Dickinson), Ab-anti CD14-PE/CD-45-FITC [Leukogate], CD3-PE and CD25-PE,CD69-PE for identify activated γδ T cells. *Results.* In 18 of NHL patients (pts) γδ T mean% in bone marrow was 4,38±3,9 and was significantly (p=0,04) higher than in peripheral blood: 3,04±1,88. Similarly γδ T CD25+% and γδ T CD69+% in bone marrow: 0,29±0,28 and 1,56±2,46 were also significantly (p=0,006 and p=0,01) higher than in blood: 0,1±0,1 and 0,64±1,21.Two possitive correlations were found: between γδ T CD25+ and γδ T CD69+ cells percentage in bone marrow and blood: r=0,53, p=0,02 and r=0,54, p=0,04. After 4 cytostatic cycles 8 of 18 pts received disease regression: complete, partial remission or stabilization (group R) and 10 of 18 pts had lymphoma progression (group P). Despite statistical significance a favorable trend was observed, that bone marrow all γδ T cells, activated γδ T CD25+ and γδ TCD69+ mean% in group R : 5,6±5,52; 0,36±0,33 and 2,31±3,49 were higher than in group P: 3,4±1,64; 0,23±0,23 and 0,96±0,99, respectively. Overall survival time (OS) in group R: 12-26 month, 16,8±5,0 was higher than in group P: 4-22 months,13,3±6,6. Two possitive correlations between bone marrow γδ T CD25+ and γδ T CD69+ percentage and OS were found, r=0,54, p=0,04 and 0,53, p=0,05 respectively. *Conclusions.* In NHL γ-δ T lymphocytes activation by tumour antigens occurs first in bone marrow than in peripheral blood, because higher γδ T CD25+ and γδ T CD69+ cells mean percentage in bone marrow than in blood were observed. Moreover, higher bone marrow γδ T CD25+ and γδ T CD69+ lymphocytes percentage at the time of diagnosis can be used as a good prognostic marker in NHL patients.

0719

EXPRESSION OF A FEW T CELL ANTIGENS AND EARLY T ORIGIN OF T-ALL/LBL PREDICT FOR POOR SURVIVAL OF ADULT PATIENTS TREATED ON ALL PROTOCOLS

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Background. Immunophenotype subtypes of T-lineage ALL (early, thymic, and mature) are conventionally used for risk stratification and selection of post-induction therapy. It is less clear if the same risk factors apply to T-cell lymphoblastic lymphoma patients (T-LBL) treated with the ALL strategy. *Aim.*To assess the prognostic value of immunophenotype patterns and clinical features of adult pts with precursor T-cell leukemia or lymphoma (T-ALL/LBL) treated with the ALL protocol. *Methods.* From 1997 to 2003 35 adult patients with T-ALL/LBL were treated according to the GMALL (German Multicenter Study Group for Adult ALL) 05/93 protocol (D. Hoelzer *et al.*, Blood 2002; 99:4379). Immunophenotype was determined by flow cytometry of cells from the lymph node, mediastinal tumor or bone marrow. Subtypes definition: early - cCD3+ (cytoplasmic), thymic (cortical) CD1a+, mature sCD3+ and CD1a-. Assessment of the panT-cell CD antigens (pT-CD) expression included: CD1a, CD2, sCD3, CD4, CD5, CD7, CD8. Survival rate was calculated by Kaplan-Meier method and compared using longrank test. Prognostic factors were analyzed by Cox's model. *Results.* Patient characteristics: males - 26 (74%), age < 35 - 30 (86%), median WBC - 18 G/L, ALL (>25% BM+) - 14 (40%), LBL - 21 (60%), mediastinal mass - 31 (88.6%), primary CNS - 4 (11%). Immunophenotype: early - 16 (45.7%), cortical - 17 (48.6%), mature - 2 (5.7%). 17 pts (48.6%) expressed CD1a antigen. Differential expression of panT-antigens: 0-3 antigens - 35.3%, 4-5 antigens - 26.5%, and 6-7 antigens - 38% of pts. The median follow up for surviving patients was 45 months. 5-year overall survival (5yOS) for all 35 pts was 42.5%, for T-LBL - 47.1%, and for T-ALL - 34.2% (p=0.99). Complete remission (CR) rate was 83% (29/35). Disease free survival for 29 CR pts was 60.3%, for T-LBL - 58.8% and for T-ALL - 63.6% (p=0.59). Patients with cortical or mature subtype had better 5yOS than those with early subtype: 56% vs 18% (p=0.036). 3yOS advantage of CD1a+ pts vs. CD1a- pts (64% vs. 39%) was not significant (p=0.075. Expression of ≥ 4 pT-CD markers vs. < 4 antigens correlated with better survival p=0.015). 5yrOS for 0-3 pT-CD - 0%, 4-5 pT-CD - 50%, and for 6-7 pT-CD - 60%. On the multivariate Cox's analysis of clinical and immunophenotypic features only female sex was predictive of poor survival (HR 6.54; p=0.002). Female sex and age > 35 correlated with progressive disease (p=0.007, p=0.046). *Conclusion.* Adult pts with precursor T-cell leukemia or lymphoma treated with the GMALL 05/93 protocol have similar outcome. Early T phenotype is a poor risk factor in both leukemic and non-leukemic patients. Expression of 3 or less panT antigens is predictive of dismal outcome.

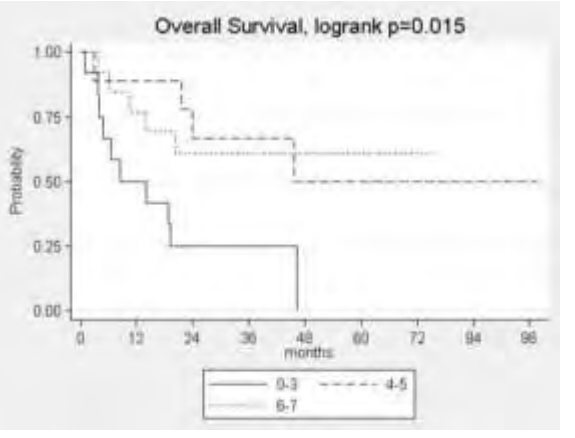

Figure 1. Number of pant/cd marker number of pant/cd markers.

0720

RITUXIMAB-CHOP EVERY 14 DAYS IN NAIVE PATIENTS WITH DIFFUSE B-LARGE CELL LYMPHOMA

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Introduction. Some studies have shown that patients with aggressive lymphoma may benefit from dose intensified schedules as CHOP-14. The addition of rituximab (R) improves response rate and survival. The support with G-CSF in dose intensification regimes may provide a good complementation of courses and advantage compared with schedules standard-dose as R-CHOP. Purpose: To evaluate the efficacy of R-CHOP-14 in naïve patients with diffuse B- large cell lymphoma (DBLC) (REAL classification). *Design.* observational, prospective and multicentric trial in a consecutive and previously untreated patients diagnosed of DBLC CD20+. Exclusion criteria: HIV positivity, other malignancies and CNS involvement. Patients and *Methods.* Since June 2003 to January 2006, 51 patients were included in an R-CHOP regimen administered every 14 days (8 courses). At baseline assessment: clinical and physical exam, blood counts, serum and urine biochemistry, albumin, β2-microglobuline and LDH level, body scan, bone marrow biopsy. Patients were classified according ECOG, clinical stage and IPI. All patients receiving prophylaxis with haematopoietic factors. Re-staging studies were performed every 4 cycles. Responses were classified as complete remission (CR), partial remission (PR), and non response (NR). Statistical analysis: Overall survival (OS), relapsed free survival (RFS). Survival analysis was performed using Kaplan-Meier and Cox regression. *Results.* Mean age 54 y (20-78), male 66.6%. ECOG 0(20), 1(17), 2(11), 3(3). B symptoms 55%. IPI score 0(3), 1(16), 2(11), 3(13), 4(7), 5(1), stage I(3), II(9), III(12), IV(27). Bulky disease 12 patients, only extranodal location 4, with extranodal location 31, haemoglobin<10 g/dL 12, albumin<3 g/dL 15, high LDH 34, high β2-microglobuline 30. After 4 cycle: 47 valuables patients (92.1%); response: 43(91.4%), 12 CR (25.5%), 31 PR (65.9%), 4 NR. After 8 cycle: 34 valuables patients (66.6%), 32 CR (94.1%), 1 PR, 1 NR. 3 patients have relapsed (5.9%) and 12 died (23.6%) (progression 6, infection 5: > 70 years 4). Adverse events 187 episodes: myelotoxicity 72% (grade 3-4 neutropenia and thrombocytopenia were observed in

80% and 16% respectively), infection 12%, gastrointestinal (5%), others (10%). Mean OS was 31 months and mean RFS 40 months. *Conclusions.* A high response rate to R-CHOP 14 in adults naïve DBLC patients (94.1%) was observed in this study with acceptable toxicity. No differences in response were observed according to age groups but higher myelotoxicity and adverse events was present in older than seventy

0721

INFLUENCE OF SERUM VASCULAR ENDOTHELIAL GROWTH FACTOR UPON TUMOR PROGRESSION IN PRIMARY SMALL INTESTINE LYMPHOMA

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The aim of this study was attempted to clarify the relationship between the serum vascular endothelial growth factor (VEGF) and clinicopathological characteristics in patients with primary small intestine lymphoma. *Materials and Methods.* The 30 patients with primary small intestine lymphoma ranged age was from 34-72 years (mean, 56 years) and included 21 men and 9 women. All cases satisfied the criteria for primary gastrointestinal lymphoma. All histologic materials were obtained by endoscopic biopsy, surgery. Immunophenothyping were assessed by monoclonal antibodies. After dividing the cases into either B-cell or T-cell phenotypes, B-cell lymphomas were classified according to the Revised European-American Classification of Lymphoid Neoplasms. The stage of tumor was classified according to modification of Musshoff *et al.* of the Ann Arbor staging system. Serums were assayed for VEGF quantitative sandwich ELISA. The minimum detectable level of VEGF was 9 pg ml-1. Analysis of differences in VEGF levels between two groups of various prognostic factors was performed with Mann-Whitney U test. The posttreatment survival probability of the patients was calculated by Kaplan-Meier method in all 30 patients and compared with the log-rank test. *Results.* The serum VEGF levels were significantly higher in patients with colorectal and/or gastric involvement than those who did not (713±0,17 pg mL-1 vs. 314±0,2 pg mL-1, $p<0.001$), in patients with diffuse infiltration under macroscopy than those who did not (789±0,15 pg mL-1 vs. 404_0,15 pg ml-1, $p<0.001$), in patients with high grade histology than those who did not (767±0,19 pg mL-1 vs. 367±0,06 pg mL-1, $p<0.001$) and in patients with perforation than those who did not (779±0,2 pg mL-1 vs. 389±0,14 pg mL-1, $p<0.001$). Those patients with MALT type tumors, less advanced stage of disease, B-cell phenotype had significantly lower serum VEGF levels. The high serum VEGF levels were significantly associated with poor survival. *Conclusion.* The high serum VEGF levels (>575 pg mL-1) appears to have poor prognosis among patients with primary small intestine lymphoma. Our study may provide a basis for the better evaluation of biological characteristics and a new therapeutic strategy.

0722

D-PACE REGIMEN: AN EFFECTIVE CHEMOTHERAPY REGIMEN TO CYTOREDUCE REFRACTORY AND EXTENSIVELY PRETREATED LYMPHOMAS BEFORE ALLOGENEIC STEM CELL TRASPLANTATION

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Background. Lymphoma patients who relapse after several lines of chemotherapy or after autologous stem cell transplantation (SCT) have a very poor prognosis with a long-term survival <15%; currently there is no standard salvage chemotherapy regimen for this subset of patients. *Aims.* The objective of the study was to assess the efficacy of DT-PACE, originally used in multiple myeloma as salvage chemotherapy regimen in patients affected by relapsed lymphoma. This regimen takes advantage of the continuous infusion (CI) principle; we chose the D-PACE regimen (without Thalidomide), in which doxorubicin is administered as a continuous infusion over 72 h, in order to: 1) cytoreduce the disease in patients eligible for an allogenic SCT 2) overcome the MDR-1-mediated resistance in tumor cells by continuous exposure at low drug concentrations 3) reduce the risk of cardiotoxicity in patients that were heavily pretreated with CHOP-like regimens. *Methods.* D-PACE regimen consisting of Dexametazone 40 mg days 1-4 i.v., Cisplatin 10 mg/ms CI days 1-4 i.v., Doxorubicin 10 mg/ms CI days 1-4 i.v., Cyclophospamide 400 mg/ms CI days 1-4 i.v., Etoposide 40 mg/ms CI days 1-4 i.v. In responding patients the regimen was planned for four cycles. Between June 2001 and July 2005 40 patients affected by relapsed or refractory lymphoma entered the study: 20 patients with Non-Hodgkin Lymphoma (NHL) (16 aggressive and 4 indolent), and 20 patients with Hodgkin'disease (HD). Median age was 40 years (range 17-75). All patients were heavily pretreated: the median number of previous chemotherapy regimens was 3 (range 1 to 6). Fifteen patients had failed a previous autologous SCT and 7 an allogeneic SCT. *Results.* No treatment-related deaths or serious adverse events occurred. An objective response was observed in 17 patients (43%); 6 complete remission (15%), 11 partial remission (28%). HD patients had a better OS compared to NHL patients ($p=0.004$), while no difference was seen in PFS between HD patients and NHL patients ($p=0.46$). Seventeen patients (7 NHL and 10 HD), who were in complete (30%) or partial remission (30%) after D-PACE courses, underwent an allogeneic SCT. Allotransplanted patients had a significantly better survival, if compared with the cohort of patients not eligible for allogeneic SCT (23 cases). In fact we observed an OS at 2 years of 54% and 27%, respectively ($p=0.029$) and a PFS at 2 years of 36% and 11%, respectively ($p=0.0006$). *Summary/Conclusion.* The D-PACE regimen is an effective and very well tolerated chemotherapy, that can be used in extensively pretreated patients with relapsed or refractory lymphoma, as debulking therapy before allogeneic trasplantation.

0723

PROGNOSIS VALUE OF POSITRON EMISSION TOMOGRAPHY USING FLUORINE 18-FLUORODEOXYGLUCOSE IN THE SETTING OF ASCT IN PATIENTS WITH HIGH GRADE LYMPHOMA

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Background. Positron emission tomography using fluorine 18-fluorodeoxyglucose ([18F]FDG-PET) is currently performed to evaluate the response in high grade non-Hodgkin lymphoma and Hodgkin's disease. However, only limited data are currently available to evaluate its prognosis value in patients with DLBCL treated with autologous stem cells transplantation (ASCT). *Aims.* The aim of the present study is to evaluate the prognosis value of [18F]FDG-PET performed immediately before and 3 months after ASCT in patients treated for DLBCL or aggressive lymphoma. Therefore, in this single-center study we retrospectively analysed a cohort of 49 patients treated between 2002 and 2005. *Methods.* Patients were 26 males and 23 females; median age was 51 years [17-65]. Patients had a diagnosis of diffuse large B-cells (n=44, 89%), mantel cells lymphoma (n=4), and anaplasic T lymphoma (n=1).

Table.

	-/-	-/+	+/+	+/-
Total (n=)	23	1	17	8
Disease evaluation after ASCT (CHESON's criteria)				
➢ Relapse or progression	0	0	2	0
➢ PR	1	1	15	2
➢ CR	22	0	0	6
Last follow-up				
➢ Alive in CR	20	0	2	8
➢ Alive in PR	0	1	12	0
➢ Alive in relapse	1	0	0	0
➢ Death from disease progression	0	0	3	0
➢ Death from other causes	2	0	0	0

The International prognosis index (IPI) value was 0-1 in 18 cases (39%), and 2-3 in 31 cases (61%), respectively. All patients with 0-1 IPI had a bulky disease, stage IV or less than complete response after the initial chemotherapy regimen. ASCT was performed as part of the first-line therapy in poor risk patients (n=39, 79.6%) or at the time of relapse (n=10). Conditioning regimen consisted of BEAM (n=43), of melphalan alone (n=2) and total body irradiation associated with chemotherapy (n=4). In order to evaluate the response, [18F]FDG-PET was performed systematically before and 3 months after ASCT. The median follow-up of living patients is 12 months [3-43 months].

Results.
-/- : [18F]FDG-PET negative before and after ASCT
-/+ : [18F]FDG-PET negative before and positive after ASCT
+/+: [18F]FDG-PET positive before and after ASCT
± : [18F]FDG-PET positive before and negative after ASCT
Conclusions. This study allows demonstrating that: i) a negative [18F]FDG-PET before ASCT has a good prognosis value; ii) as expected, a positive [18F]FDG-PET before and after ASCT is associated with a poor outcome; iii) interestingly, patients with positive status before ASCT and negative status after ASCT have a prognosis similar as those negative before and after ASCT (under reserve because of the low number of patients and the short follow-up).

0724

LONG TERM SURVIVAL DATA OF PEDIATRIC NHL PATIENTS

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Background. Evaluating NHL treatment result data can provide insight and useful information to guide our future approach and possibly improve the care of our children. *Aims.* The characteristics of the patients with NHL, treated in our Department over the last 15 years are analyzed. The results are summarized in total and by the different time course of presentetion and treatment schedule. *Methods.* From 1990 to 2005, 47 children (10 girls) were diagnosed with NHL. Mean age at diagnosi was 8,40 years (range, 0,33 to 14,5). During the 1st, 2nd and 3rd decade 14 (3), 17 (3) and 16 (4) patients (girls) were diagnosed, respectively. Based on pathology, B-NHL, T-NHL and Ki-1 (+) NHL was diagnosed in 31, 11 and 5 patients, respectively. Most common presenting sites were the mediastinum (15), the neck area (12) and the abdomen (8). For all patients, stage I, II, III, IV was found in 3, 14, 23 and 7 patients, respectively. Treatment varied through the last 15 years. The approach of the BFM protocol was applied since 1995 (BFM-NHL 90 and from 1997 the BFM-NHL 95 protocol). Irradiation was given to 5/47 patient (with B-NHL 2/5 and with T-NHL 3/5) and autologous SCT to 4 patients, all with B-NHL (1 with CNS disease, 1 with residual disease at the end of treatment and 2 at relapse). *Results.* Thirty eight (38) patients are alive: 35, 2 and 1 in 1st, 2nd and 3rd remission, respectively. Nine (9) patients in total have succumbed (2 died soon after admission from other hospitals due to acute phase complications and 5 patients died during the 1st decade of our retrospective study (with T- histology and extensive disease). EFS (35 of 47 patients) is 74,4% and OS (38 of 47 patients) is 80,9%, for a median follow-up time of 6,1 years (range, 0,01 to 14,7) for all patient. For the 34 patients treated with the BFM-95 protocol since 1997, EFS and OF is 79,4% and 88,2%, respectively, for a median follow-up time of 4,8 years. *Conclusions.* Overall and events free survival and outcome of our patients with NHL treated during the last 15 years is standing high . Due to continuous improvement of the supportive care and understanding of the protocol philosophy while by implementing the BFM NHL treatment approach for our patients the mentioned high standing outcomes have been documented. There has been limited use of irradiation and stem cell transplantation.

0725

PREVALENCE OF HEPATITIS B IN PATIENTS WITH HODGKIN AND NON-HODGKIN'S LYMPHOMAS

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Introduction. High prevalence of hepatitis B infection has been observed in patients with lymphomas in previous studies, but it is still not clear whether there is an association between malignant lymphomas and hepatitis B virus (HBV). *Aim.* The aim of this study was to investigate the incidence of hepatitis B amongst patients with Hodgkin and non-Hodgkin lymphoma. *Patients and Results.* We retrospectively studied 1191 patients with lymphoma who were admitted to our hospital unit from January 1980 to December 2005. They consisted of 404 cases of Hodgkin lymphoma (HL) and 787 cases of non-Hodgkin lymphoma (NHL). Patients were tested for hepatitis B antigen (HBAg) during their first admission to the hospital. Nine out of 404 patients with HL (2.23%) and 46 out of 787 patients with NHL (5.84%) had positive HBAg. The rate of hepatitis B infection in patients with HL and NHL was higher than the general Greek population (0.9%). When compared statistically by the x2 test the prevalence in patients with HL was not significantly higher than normal persons (p=0.055), while the prevalence in patients with NHL was significantly higher than the general population (p<0.001). HBV infection is known to cause immune disorders and clonal expansion of B lymphocytes, probably contributing to lymphomagenesis. In addition the immunodeficiency that preexists and leads to chronic hepatitis B may also be a predisposing factor for the development of a malignant lymphoma. It is not known whether patients with hepatitis B have the same response rates to treatment and survival rates with the rest of the patients. Larger series of patients are needed to investigate it. *Conclusions.* We observed high rate of HBAg in patients with lymphomas especially NHL. Hepatitis B virus may play a role in lymphomagenesis. Further studies are required to clarify the association between HBV infection and malignant lymphomas.

0726

SAFETY AND EFFICACY OF RITUXIMAB COMBINED WITH CHEMOTHERAPY FOR LYMPHOMA DURING PREGNANCY

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Background. Management of non-Hodgkin lymphoma during pregnancy remain a difficult challenge for both patients and doctors. Treatment may allow to obtain a complete response for the mother without side effects for the fetus. Few data have been published on the safety of rituximab during pregnancy. Rituximab is a chimeric IgG1 antibody, which can cross the placenta and interact with fetal B cells. *Aims.* We report the case of a woman with a diffuse large B cell lymphoma during pregnancy who was treated with rituximab. *Methods.* A 28 year old woman was diagnosed with CD20+ diffuse large B cell lymphoma in her 18 week of pregnancy. Staging show a stage II with mediastinal bulky. After careful consideration and patient informed consent, the patient was treated with a combination of rituximab and chemotherapy with standard CHOP: rituximab 375 mg/m^2 D1, cyclophosphamide 750 mg/m^2 D1, doxorubicin 50 mg/m^2 D1, vincristin 2 mg D1, and oral prednison 100 mg D1 to D5 given in 3-week cycles. There were no infusions reactions. During treatment, the intrauterine development was closely monitored. She received four cycles of treatment before delivery. She delivered in the 33 week of pregnancy and in very good partial remission, a 2000 g healthy child via caesarean section. Two weeks after caesarean, she was treated with two another cycles of chemotherapy with rituximab. Tep scan concluded to a complete remission. The child is now 10 month old and has a completely normal growth. After a follow up of 10 months, our patient is still in complete remission. *Results.* Little is know about the safety and efficacy of rituximab during pregnancy. To our knowledge, there are three cases treated with rituximab during the first (one patient) or second (two patients) trimester of pregnancy. Rituximab seems safe and without significant consequences for the fetus. Although B cells were extremely low at birth and during first weeks in the child, no infectious complications have been reported. *Conclusions.* Combination of rituximab with chemotherapy is safe and might be a valuable treatment option for pregnant women with CD20+ lymphoma. Controlled studies are necessary to confirm this data.

0727

FALSE POSITIVE PET FINDINGS IN NHL PATIENTS AFTER CHEMOTHERAPY RELATED TO MACROPHAGE-RICH LESIONS

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PET (Positron Emission Tomography) imaging uses the glucose analogue 18F-FDG as a tracer, and is an excellent method to detect small focal sites of high metabolic activity, which are frequently indicative of tumors. However, 18F-FDG uptake is not tumor specific. Various forms of inflammatory lesions and healing tissues, that have a high concentration of inflammatory cells (neutrophils, activated macrophages), also take up 18F-FDG, and are a major cause of false positive *Results.* 18F-FDG PET represents a major advance in both staging and restaging of Non Hodgkin Lymphoma (NHL), however the sensitivity in the setting of restaging is lower, and is associated with a significant number of false positive findings. Here we describe two cases, that illustrate the caution needed in the interpretation of PET scans in the restaging context of NHL patients. *Case 1.* A 57 year old male, with a solitary nodule with 10 cm of diameter, localized to the segment IV of the liver, histologically compatible with Diffuse Large B-Cell NHL (stage IE A). After treatment with R-CHOP, we observed in CT scan, a 60% reduction of the hepatic lesion dimensions. The PET/CT showed increased 18F-FDG uptake in the segment IV of the liver, compatible with the persistence of NHL. A left hepatectomy and resection of the segment IV was performed. The histological examination of the surgical specimen showed a large nodular area of tissue necrosis, surrounded by a fibrosis capsule and numerous activated macrophages, but no signs of persistent disease. One year after surgery, the patient is in complete remission. *Case 2.* A 53 year old male, with Diffuse Large B-Cell NHL, stage II A, bulky (infra-diaphragmatic), treated with R-CHOP. After treatment, a persistent thickening of the mesenteric fat was seen in the CT scan. The PET/CT showed increased 18F-FDG uptake in multiple abdominal confluent masses, in the pre-aortic and mesenteric regions, compatible with the persistence of NHL. A laparotomy with multiple biopsies was performed. The histological examination of the surgical specimen showed large areas of fat necrosis, and no evidence of NHL. In conclusion, 18F-FDG PET is an essential tool in the management of NHL patients. However, in case of positive 18F-FDG-PET findings, histological confirmation is required, in order to exclude post treatment benign inflammatory lesions, and to avoid unnecessary treatment approaches.

Myeloma and other monoclonal gammopathies III

0728

DIVERSE NICHES WITHIN MULTIPLE MYELOMA BONE MARROW SAMPLES AFFECT PLASMA CELL ENUMERATION AND FCM PROFILE

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Background. The diagnosis of MM is based on the combination of clinical and laboratory criteria including bone marrow (BM) morphological assessment (percentage of plasma cells counted), often combined with flow cytometry (FCM). *Aims.* In this study we compared the bone marrow plasmocytosis by microscopic examination of BM aspirates, to the FCM results in samples obtained form MM patients. We also tested whether the noted discrepancy between these two methods applies only to MM, or represents a trend in other hematopoietic malignancies as well. *Methods.* The number of plasma cells in BM aspirates from 41 MM patients were analyzed simultaneously by morphological evaluation and by FCM using the following panel of antibodies: CD38, CD45, CD56, CD117, CD138, and IgG isotype controls. Each sample was assessed independently by two qualified laboratory specialists and/or hemato-pathologist. Seven BM samples from patients with acute myeloid leukemia (AML) were compared in a similar manner. *Results.* In MM it was evident that FCM under-estimated the number of BM plasma cells samples by an average of 60%, compared with conventional morphological evaluation. On the other hand in AML there was a good correlation between the morphological and FCM assessments of the blast cell population, indicating that the discrepancy observed in the MM BM samples may be related to unique characteristics of the malignant plasma cells. This discrepancy may results partially due to the fact that bone marrow aspirates contain cells associated with the lipid-enriched spicules, while flow cytometry analysis is performed on the bone marrow fluid which is depleted of these fat tissue -adhesive plasma cells. When disrupted spicules from MM BM samples were isolated (by repeated passages through 21g needle), a 40% increase in the plasma cell percentage was noted, compared with the fluid of the same BM samples. In order to determine the FCM profile of the cells in these two fractions, we isolated BM derived spicules from aspirates of MM patients, and either sheared them mechanically with repeated passages through a 21g needle, or treated them with a cocktail of three extracellular matrix (ECM) degrading enzymes (heparinase I, chondroitinase ABC and hyaluronidase), followed by mechanical shearing. Only a combination of these two methods (shearing and ECM degrading enzymes) released the highly adhesive plasma cells from the spicules. The released myeloma cells displayed a different FCM profile and in particular had a higher level of CD138 expression. *Summary.* We have shown a major discrepancy between the percentage of MM cells obtained by routine BM morphology and flow cytometry counts. It is possible that this discrepancy is partially attributable to the two distinct microenvironmental components occupied by MM cells in the BM sample - the lipid spicules, and the fluid phase. MM cells located in the different niches of the BM also differ in their FCM profile. This study indicates that multiple myeloma patients contain heterogeneous populations of malignant plasma cells. These sub-populations may play distinct roles in the different biological and clinical manifestations of the disease.

0729

COMBINATION OF BORTEZOMIB, MELPHALAN, PREDNISONE AND THALIDOMIDE IN ADVANCED MYELOMA: A PHASE II CLINICAL TRIAL

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Background. Bortezomib (Velcade™) and Thalidomide are effective for the treatment of refractory multiple myeloma (MM). *in vitro* studies showed that Bortezomib can restore sensitivity to Melphalan-resistent MM cell lines [Clin. Cancer Res 2003; 9:1136-1144]. In newly diagnosed patients (pts), the addition of Thalidomide to the standard oral Melphalan/Prednisone combination significantly increased response rate and event free survival [Cancer 2005;104:1428-33]. *Aims.* A phase II trial was

initiated to evaluate the efficacy and the safety of the combination therapy of Velcade™, Melphalan, Prednisone, Thalidomide (VMPT) in advanced myeloma. *Methods.* Oral Melphalan was administered at 6 mg/m^2 on days 1-5, oral Prednisone at 60 mg/m^2 on days 1-5 and Thalidomide at 100 mg/day continuously. Velcade™ was administered by IV bolus on days 1, 4, 15, 22 at three dose levels: in the first cohort (10 pts) at 1.0 mg/m^2; in the second cohort (10 pts) at 1.3 mg/m^2 and in the third cohort (10 pts) at 1.6 mg/m^2. Each course was repeated every 35 days for a total of 6 courses. Dose Limiting Toxicity (DLT) was defined as the occurrence of any grade 3-4 non hematological toxicities, a grade 4 neutropenia > a week, or any grade 4 hematological toxicity except neutropenia. *Results.* Thirty pts with relapsed or refractory myeloma were enrolled, median age 66 years (range 38-79), 67% IgG, 17% IgA, 17% Bence Jones. The median β2 microglobulin was 3.4 mg/L (range 0.4-11.8). Fourteen pts received V-MPT as second line therapy, 16 as third line. Twenty pts received prior autologous transplant, 10 conventional chemotherapy and 9 thalidomide-based regimens. After a median of 5 courses, 20 pts (66.7%) achieved an objective response (complete response 16.7% and partial response 50%). Furthermore, 2 pts (6.7%) achieved a minimal response and 3 (10%) s; le disease. Five pts (16.7%) were refractory to treatment and experienced progressive disease. In the first cohort, 3 DLT were observed (grade 3 pneumonia, grade 3 febrile neutropenia and grade 3 vasculitis); in the second cohort, 5 DLT were observed (grade 3 Herpes Zoster infections, grade 4 thrombocytopenia and grade 4 anemia); in the third cohort 5 DLT were observed (grade 4 thrombocytopenia, grade 3 fatigue, sensory neuropathy grade 3, grade 3 Candida esophagitis). The most common grade 1-2 toxicities were: infections, fatigue, peripheral neuropathy and constipation. After introduction of prophylaxis with acyclovir, no new HZV reactivation was observed. Among the 8 pts with baseline peripheral grade 1 neuropathy before VMPT treatment, 5 worsened (one grade 3). Treatment-related neuropathy developed *de novo* in 4 pts (one grade 3). *Conclusions.* Initial results showed that VMPT is a promising regimen for advanced myeloma.

0730

SERUM CONCENTRATIONS OF DICKKOPF-1 PROTEIN ARE INCREASED IN PATIENTS WITH MULTIPLE MYELOMA AND REDUCED AFTER AUTOLOGOUS STEM CELL TRANSPLANTATION

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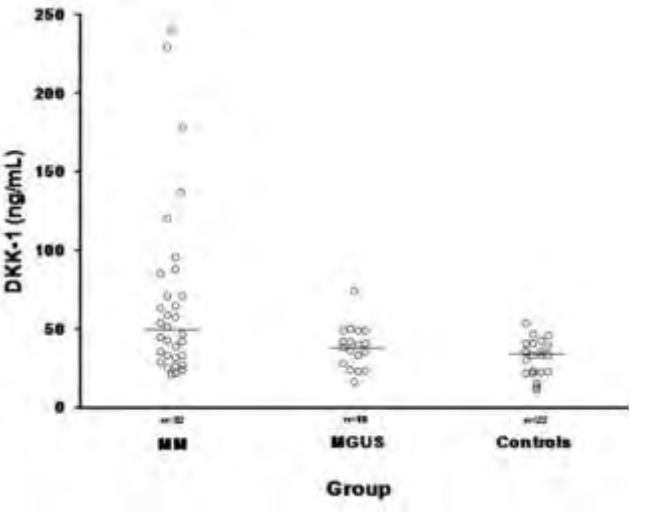
Background. Dickkopf-1 (DKK-1) protein, a soluble inhibitor of Wnt signalling, has been implicated in the pathogenesis of myeloma bone disease. DKK-1 protein was detected in plasma cells isolated from myeloma patients with bone lesions but not in normal plasma cells or in plasma cells isolated from MM patients with no lytic disease. However, it is unclear whether serum DKK-1 concentrations are elevated and whether this is related to other markers of bone and/or tumour development in myeloma (MM). *Aims.* The aim of the study was to evaluate, for the first time, circulating serum DKK-1 concentrations in MM patients at diagnosis, before and after autologous stem cell transplantation (ASCT) and in patients with monoclonal gammopathy of undetermined significance (MGUS) and examine possible correlations with clinical data. *Methods.* We studied 32 patients with MM at diagnosis, 18 MM patients pre- and post-ASCT, 18 patients with MGUS, and 22 healthy controls of similar age and gender. Evidence of bone involvement was documented using plain radiography. A series of serum bone remodelling indices: i) bone resorption markers [NTX and TRACP-5b], ii) bone formation markers [bone-alkaline phosphatase and osteocalcin], and iii) osteoclast stimulators [soluble RANKL and osteoprotegerin] were determined by ELISA. DKK-1 serum levels were also determined by ELISA using a goat anti-human DKK-1 antibody (R&D Systems, Abingdon, UK). *Results.* The serum DKK-1 concentration in MM patients at diagnosis (mean±SD: 67±54 ng/mL), were increased compared with MGUS patients (38±13 ng/mL; p=0.006) and controls (31±11 ng/mL; p=0.02) [Figure]. There was no statistically significant difference between MGUS patients and controls. Patients with stage 2 and 3 myeloma, according to the novel ISS, had higher values of DKK-1 than patients with stage 1 disease [median values of DKK-1 for stages 1, 2 and 3 were: 38.5, 55.4, and 60.8 ng/mL, respectively; p (stage 1 vs. stage 2) = 0.014, p (stage 1 vs. stage 3) = 0.04, and p (stage 1 vs. stages 2+3) = 0.005]. There was no correlation between serum DKK-1 concentrations and the extent of bone disease; however, this may reflect the numbers available in the present study, or the limitations of the technique used to detect the osteolytic lesions (plain radiography). There was also no correlation between serum levels of DKK-1 and bone remodelling markers. Before ASCT, DKK-1 serum levels were increased in myeloma patients compared with controls (63±77 ng/mL vs. 31±11 ng/mL; p=0.03). Over time there appeared to be a sustained decrease in DKK-1 levels after ASCT (p=0.04). In contrast, the markers of bone formation, OC and bALP were increased. *Conclusions.* Serum DKK-1 is increased in MM patients and correlates with stage of the disease using the new ISS; furthermore, the reduction of DKK-1 levels after ASCT may be associated with the normalization of osteoblast function, as assessed by bone formation markers. These results, if reconfirmed in a larger series of patients, could provide the basis for developing drugs that block DKK-1, thus restoring osteoblast function, and counteracting the increased osteoclastogenesis observed in MM.



0731

DEVELOPMENT OF A NOD-SCID HU ANIMAL MODEL TO INVESTIGATE WALDENSTRMS MACROGLOBULINEMIA*

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Waldenstrom's macroglobulinemia (WM) is a B-cell lymphoproliferative disorder characterized by predilection for bone marrow involvement and secretion of IgM paraprotein. The purpose of this study is to establish an animal model mimicking closely the disease in humans. We implanted into NOD-SCID mice human cancellous bone obtained from adults undergoing total hip arthroplasty or hemiarthroplasty. Cancellous bone was harvested in compact cores from the femoral head and was implanted in the hindlimb muscles of ten NOD-SCID mice. Mice were used when 6 to 8 weeks of age (25-30 grams). The size of the bone implant was between 16 and 22 mm^3. Eight to twelve weeks after the bone implantation, 3-5×10^6 WM cells freshly harvested from a WM patient were injected i.m. very close to the bone implant into 4 mice, and 1×10^6 cells from the same WM patient were injected i.v. into the tail vein of 2 mice bearing human bone implants. Also, two freshly harvested bone marrow (BM) core biopsies from a patient with active WM were implanted as described. All animals had a human bone fragment from non WM individuals in the opposite hindlimb. Tumor progression was determined by monitoring human immunoglobulin M (IgM) levels in murine plasma. Immunohistopathologic evaluation was performed on the human bone grafts, and murine tissue including the femurs, and tibia, the brain, liver, spleen, lung and kidney. One out of four mice injected i.m. into the bone fragment vicinity with WM cells showed elevated levels of human IgM indicative of the development of the disease. One out of two i.v. injected mice had elevated IgM one month following the injection of the WM cells. Both mice implanted with the bone marrow core biopsies showed a declining level of IgM directly after the implantation of the biopsy, but 3 months following the implantation IgM started increasing and reached levels above baseline. Histopathologic analy-

sis was performed using antihuman reagents for expression of CD20 and IgM. Positive cells for both CD20 and IgM were found in the BM core biopsies from the WM patients and the human bone graft opposite to the injected/implanted site. The stain was present in the cytoplasm and/or the surface of the positive cells. Murine tissue needs further histopathologic evaluation. Mice may need to be followed for more extended periods of time to fully assess the pattern of WM growth in this model. In conclusion, this SCID-hu WM model more closely resembles the human disease. It differs from the recently created WM model by Tassone *et al.*, (2005), since the utilization of adult bone compared to fetal, along with the implantation of WM bone biopsy, allows us to study the biology of the malignant cells in their native BM microenvironment.

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0732

COMBINATION OF BORTEZOMIB AND DEXAMETHASONE FOR PATIENTS WITH AL AMYLOIDOSIS

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Background. Primary systemic amyloidosis (AL) is a clonal plasma cell dyscrasia and is characterized by widespread deposition of abnormal amyloid fibrils derived from abnormal light chains, leading to multisystem organ failure. Aggressive treatment of AL amyloidosis with high-dose melphalan and autologous stem cell transplantation (HDM-ASCT) is currently the treatment of choice for selected patients, while the combination of melphalan and dexamethasone is used for patients who are not eligible for HDT. Bortezomib is a proteasome inhibitor with proven activity in relapsed/ refractory Multiple Myeloma, alone or in combination with dexamethasone. *Aims.* To evaluate the activity and feasibility of the combination of Bortezomib and Dexamethasone (BD) in patients with primary systemic amyloidosis. *Methods.* We treated consecutive patients with histologically proven, symptomatic AL amyloidosis who had measurable disease, defined as a serum M-spike >0.5 g/dL or urine M-spike>200 mg/24 hours or involved immunoglobulin free light chain (FLC) ≥100 mg/L and an abnormal FLC ratio. None of the patients had a history of multiple myeloma. Patients were treated with the combination of Bortezomib 1.3 mg/m^2 on days 1, 4, 8 and 11, and Dexamethasone 40 mg on days 1 to 4, every 21 days, for 4-6 cycles. Dose modifications were made based on toxicity. For the assessment of hematologic and organ response we followed the recommendations of the 10th International Symposium on Amyloid and Amyloidosis (Gertz *et al.,* Am J Hematol (2005) 79: 319-328).

Table 1. Patient characterists.

Male/Female		4/3
Age (median/range)		61 (45-79)
Light chain type k/λ		0/7
Involved FLC, mg/L (median/range)		436 (41-894)
Bone marrow Plasma cells (median/range)		20% (15-30%)
Number of Major organs involved	1	2
	2	1
	>2	4
Heart Involvement (number of patients)		5
Ejection fraction (median/range)		74% (47-84)
LV septum mm (median/range)		17 (10-20)
NYHA class >1		4
BNP>120 pg/mL		5
TnT>0.035 Ng/mL		0
β2-microglobulin>2.7 mg/dL		4
Creatinine >1.7 mg/dL		2
Creatinine clearance < 50 mL/min		2
Albumin < 3.5 g/dL		5
Urine protein (mg/24 hrs) (median/range)		2800 (1500-4950)
Alk. Phosphatase > 1.5 ULN		0

Results. Over the last 6 months, 7 patients have started treatment with BD. Their characteristics are shown in table 1. Three had at least one prior therapy, one with HDM-ASCT followed by melphalan/prednisone, one with VAD and one patient with melphalan and dexamethasone. Four patients not eligible for upfront HDM-ASCT received BD as primary treatment. Among 6 evaluable patients so far, two had a complete hematological response (CR) and 3 had partial hematologic response (PR). Hematologic response was achieved 3 to 12 weeks (median 5 weeks) after the initiation of treatment. One patient who achieved CR to BD was subsequently treated with HDM-ASCT. It is too early to evaluate organ response Toxicity was manageable; 5 patients had grade 1 orthostatic hypotension, one had grade 1 neurotoxicity and one patient had grade 2 fatigue, 4 had grade 1 edema, two patients had grade 1 diarrhea and two had grade 1 constipation. Dose reduction was needed only in one patient due to fatigue. Hematologic toxicity was minimal; grade 3 lymphopenia and grade 1 thrombocytopenia were the main toxicities. *Conclusions.* The combination of BD is feasible for patients with AL amyloidosis. Patients achieve a rapid hematologic response with manageable toxicity but additional follow up is needed to assess organ response. Further investigation is needed to explore this combination in the treatment of AL patients either in relapsed patients or in the frontline treatment of patients not eligible for HDM-ASCT.

0733

LENALIDAMIDE (REVLIMID), IN COMBINATION WITH CYCLOPHOSPHAMIDE AND DEXAMETHASONE IS AN EFFECTIVE REGIMEN FOR HEAVILY PRE-TREATED MYELOMA PATIENTS

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Background. Lenalidamide (Revlimid) is an oral immunomodulatory drug that has been shown to be effective for the treatment of relapsed refractory myeloma, and *in vitro* laboratory studies suggest that its action may be synergistic with a number of conventional chemotherapeutic agents. *Aims.* To assess the efficacy and toxicity profile when lenalidamide is used in combination with cyclophosphamide and dexamethasone for patients with relapsed refractory disease. *Methods.* Multiply relapsed patients were given Revlimid 25 mg po on days 1-21, dexamethasone 40 mg po days 1-4 and days 12-15, and cyclophosphamide 500 mg po days 1, 8, 15 and 21 of a 28 day cycle for a maximum of 6 cycles of treatment. Prophylaxis with acyclovir, septrin and proton pump inhibitors was routinely used, no patient received prophylactic anticoagulation. Toxicity profiles and response were assessed every 4 weeks. *Results.* To date 18 patients have been included in the study. All were heavily pre-treated with a median of 4 previous lines of therapies (range 2-6). 13 patients had received high dose melphalan, 16 patients thalidomide and 13 patients bortezomib. The median time from diagnosis to treatment initiation was 49 months (range 11-122). To date 50 complete courses of therapy have been given to 15 patients with median number of 3.5 courses (range 1-6). 6 patients experienced neutropenia with the neutophil count falling below 0.5×10^9/L in a total number of 14 cycles, which resulted in a dose reduction or stopping of cyclophosphamide in 5 patients. 8 patients received GCSF to maintain their neutrophil count. Only one patient required a dose reduction of Revlimid to 25 mg alternate days. Three patients required intravenous antibiotics for neutropenic fever. The side effect profile was manageable, importantly no patient experienced sedation, constipation or worsening of peripheral neuropathy. One patient with heavy myeloma load suffered a DVT, but continued on therapy achieving a PR once anti-coagulation had been commenced. 10 of the 11 patients assessable for response achieved a response with 3 VGPR, 6 PR and 1MR. The median time to response was prompt at 4 weeks (range 2-12). To date only one patient has discontinued therapy because of a failure to respond to therapy. One patient has completed 5 courses of therapy the remainder being on treatment. *Summary*. The combination of CRD is effective in heavily pretreated myeloma patients and has a manageable toxicity profile.

0734

ACQUIRED ACTIVATED PROTEIN C RESISTANCE , MULTIPLE MYELOMA AND THROMBOSIS DURING THE INDUCTION PHASE OF TREATMENT

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Background. Thrombosis is increasingly recognized as a common complication in patients with malignancy.Despite the common finding of VTE in patients with cancer, significance of coagulation test abnormalities predicting for deep venous thrombosis (DVT) still remain to be proven, although recently has been reported the impact of diagnosing acquired activated protein C resistance (APC) on DVT development in myeloma patients. Thalidomide has been used to treat refractory MM,

and an increased risk of thrombosis has been reported when it is employed in combination with other chemotherapeutic agents. *Aims.* The purpose of this study was to examine the association between chemotherapy, thalidomide and APC-R with DVT development in a cohort of newly diagnosed MM patients. *Methods.* One hundred and twenty two newly diagnosed multiple myeloma patients were evaluated. *Methods.* We designed a descriptive, retrospective , longitudinal and observational study. Patients diagnosed with deep vein thrombosis and MM were evaluated. Coagulation tests were performed in the last 60 patients including acquiered activated protein C resistance, Leiden factor V, factor VIII , serum S and C protein.Thrombosis was documented using standard criteria and diagnostic imaging.

Figure 1. Acquired activated protein C Resistance (APC-R) and Deep vein thrombosis (DV
p value 0.007, association between DVT and APC-R.

Time to thrombosis was defined as the period of time between multiple myeloma diagnosis and the thrombotic event. Statistical analyses were performed using SPSS version 10.0. Fisher's Exact test was used to evaluate the statistical significance of associations in two-way contingency tables. Cochran'Mantel'Haenszel methods were used to evaluate the association between APC resistance and DVT occurrence while controlling for thalidomide exposure. A p value <0.05 was considered as statistically significant. *Results.* The frequency of response (CR,VGPR/NCR, PR) in the group of thalidomide and dexamethasone was 80% (CR,22.8% VGPR/NCR 20% and PR, 37.2%) being higher than VAD, 50.7% (CR 16.4%,VGPR/NCR 5.9%, PR 28.4%). p 0.0005.DVT occurred in 12 patients treated with thalidomide/ dexamethasone and 6 with VAD (p 0.005). From the last 50 patients, 5 presented APC-R and 3 of them developed DVT (60%). All of them received Thal/Dex as therapy. We performed an ACPR re-test after disease response, showing that patients who presented any type of response developed a negative re-test.Patients with thrombosis in the VAD group developed the event shorter ; median time to thrombosis in this group was 2.2 months versus 4.2 months in the thalidomide plus dexamethasone group. (p 0.005) A cohort of patients is being evaluated using aspirin to prevent deep vein thrombosis during the management with thalidomide plusdexamethasone. In conclusion , it is clear that in multiple myeloma thalidomide increases the thrombotic risk, particularly in combination with chemotherapy and APC-R. APC-R appears to be a transitional condition may be related to myeloma status.

0735

INTERNATIONAL STAGING SYSTEM IN MULTIPLE MYELOMA: IS ALBUMIN TRULY NECESSARY IN THE MODEL?

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Background. The prognostic significance of the combination of β-2 microglobulin (B2M) and albumin (alb) in multiple myeloma (MM) was recently confirmed by the International Myeloma Working Group in a study of 10750 patients worldwide. They subsequently employed these two factors in order to develop the International Staging System (ISS). Weber *et al.* in an early attempt to validate the ISS in 894 consecutive patients, questioned the adding prognostic value of alb, since they demonstrated similar results using a staging system (WSS) with B2M alone in identical cut offs with ISS. *Aim.*To evaluate the necessity of alb employment in the model of ISS by applying ISS and WSS in a large number of previously untreated MM patients. *Methods.* Between January 1989 and January 2006, 470 consecutive patients were diagnosed with MM in our department. Ninety-two (19.6%) patients received high dose therapy followed by autologous stem cell transplantation and the rest 378 (80.4%) were treated with conventional chemotherapy. All patients were classified according to 1. ISS. Stage I: B2M <3.5 mg/L and alb ≥3.5g/dL. Stage II: neither stage I nor III. Stage III: B2M ≥5.5 mg/L. 2. WSS. Stage I: B2M <3.5 mg/L. Stage II: 3.5mg/L≤ B2M <5.5 mg/L. Stage III: B2M ≥5.5 mg/L. Virtually, the difference between ISS and WSS is that patients with B2M <3.5 mg/L and alb < 3.5 g/dL are classified in stage II according to ISS, while according to WSS they belong in stage I. We decided to analyze these patients separately in order to detect in which prognostic group they practically belong. Overall survival (OS) was estimated according to Kaplan-Meier method. Differences in survival were assessed using the log-rank test. *Results.* According to ISS, 135 (28.7%) patients were classified in stage I, 168 (35.7%) in stage II and 167 (35.5%) in stage III, with corresponding median OS 76 (95%CI: 66-86), 40 (95%CI: 35-45) and 23 (95%CI: 19-27) months. According to WSS, 177 (37.4%) patients belonged to stage I, 126 (26.8%) to stage II and 167 (35.5%) to stage III, with median OS 64 (95%CI: 54-74), 43 (95%CI: 38-48) and 23 (95%CI: 19-27) months respectively. Statistically significant difference in survival was detected between all stages in both staging systems (p<0.001). There were 42 patients with B2M <3.5 mg/L and alb < 3.5 g/dL, who when analyzed separately, had a median OS of 40 (95%CI: 32-48) months, having no statistically significant difference with stage II patients of either staging system (p>0.7). *Conclusions.* Both ISS and WSS achieved a homogeneous distribution of patients among the three stages and demonstrated a high discriminatory efficacy. Stage I patients according to WSS had a lower OS compared to stage I patients in ISS. This is due to the inclusion of all patients with B2M <3.5 mg/L in stage I, irrespective of their alb level, while in fact, patients with B2M <3.5 mg/L and alb < 3.5 g/dL, belong to stage II. So, alb cannot be excluded from the ISS model, since it is absolutely necessary in order to identify true low-risk patients.

0736

MOLECULAR CHARACTERIZATION OF A PANEL OF MULTIPLE MYELOMA CELL LINES: A MODEL FOR AN INTEGRATIVE GENOMICS APPROACH

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Background. The availability of Human Myeloma cell lines (HMCLs) has significantly contributed to elucidate the molecular and biological aspects of Multiple Myeloma (MM), such as the identification of the most recurrent IGH translocations and the complex network of cytokines affecting plasma cell growth and angiogenesis. Recently, genes targeted by the chromosomal translocations, as well as the activity of novel candidate specific therapeutic agents, have been investigated in HMCLs. However, it is well known that the establishment in culture *per se* and the continuous passages in culture confer to the HMCLs a progressive independence from growth factors as well as the gain of multiple genetic lesions. *Aims*. The purpose of the present study was a detailed characterization of a panel of 23 HMCLs using a genomic integrative approach combining Fluorescence in-situ hybridization (FISH) and both gene expression and genome-wide profiling. *Aims*. The most recurrent IGH translocations were determined by FISH and RT-PCR in 23 HMCLs. Gene expression profiling (GEP) of the 23 HMCLs has been generated using Affymetrix HG-U133A high-density oligonucleotide arrays. Expression data has been analyzed with unsupervised (two-dimensional hierarchical clustering) and supervised (SAM, Significant Analysis of Microarrays) *Methods.* Genome wide profiling data for 17 HMCLs has been generated on high-density SNP arrays and subsequently analyzed to investigate copy number alterations. *Results*. In the studied panel of 23 HMCLs, 8 lines displayed the t(4;14) translocation, 4 the t(11;14), 5 the t(14;16), 2 the t(6;14), 1 the t(14;20) and 13 the t(8;14), with the consequent deregulation of the respective target genes. The unsupervised analysis performed on the gene expression data showed that only t(4;14) HMCLs could be grouped in a clearly distinguishable cluster. A subset of 6 HMCLs, 4 of which without any known IGH translocations, showed the overexpression of the members of the *GAG* tumor antigens, previously described as associated to unfavourable tumor progression in MM patients. Interestingly, the GEP analysis revealed that *MAF* overexpression is not strictly related to the presence of the t(14;16), since its expression was found in cell lines negative for the translocation. In the group of HMCLs overexpressing *MAF* or *MAFB*, the specific deregulation of the known *MAF* target genes, including *CCND2* and *ITGβ7*,

was observed. Finally, our data show that all HMCLs are characterized by a complex kariotype, the most common aberrations being the gain of chromosome arm 1q and the loss of chromosome arms 1p, 13q and 17p. *Conclusions.* In the present study, we extend the characterization of most of the known HMCLs, making it possible a more accurate selection as appropriate model of MM for *in vitro* experiments and provide insights into the characterization of novel potential genetic lesion in primary tumors.

0737

MONOCLONAL GAMMOPATHY: NATURAL HISTORY STUDIED WITH A RETROSPECTIVE APPROACH

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Background. Monoclonal gammopathy of undetermined significance (MGUS) indicates the presence of monoclonal immunoglobulin in serum without evidence of multiple myeloma (MM), Waldenströms macroglobulinemia (WM), amyloidosis or other malignant lymphoproliferative disease. The prevalence of MGUS and the probability of progression to malignant lymphoproliferative disease vary between studies reflecting different populations studied and sometimes referral bias. The probability of progression from MGUS to malignant plasma cell disease is reported to be 12%, 25%, 30% at 10, 20, 30 years, respectively, in the largest series so far (Kyle *et al.* 2002). The size of the initial paraprotein and the non IgG type were the strongest predictors of progression. Other smaller studies have supported these findings. Although it is known that a significant proportion of cases with MGUS will progress to malignant disease, sometimes after a long benign phase, it has never been investigated in how many cases MM or WM have been preceded by MGUS. *Aim.*The objective of this study was to examine the natural history of monoclonal gammopathy using a retrospective approach, with a long observation period, in an effort to estimate the proportion of MM cases with a prodromal MGUS phase. *Methods.* Data were obtained from the Icelandic Cancer Registry for all MM and WM cases in Iceland since 1955 (1991 for WM) and compared with the Icelandic Heart Association's (IHA) biobank registry. Frozen serum samples were found from 66 MM cases and 10 WM cases. These samples were collected between 1967 and 1995 by IHA as part of the population-based Reykjavik Study in a nonselected manner. Protein electrophoresis (PE) and immunofixation (IF) was performed on all samples from the cases and two controls for each case, matched for age, gender and sampling time. *Results.* Paraprotein was found with PE in 28% of the samples from cases (n= 21, MM=20, MW=1) and 1.3% from controls. With IF paraprotein was found in 46% of the samples from cases (n=35, MM=32, MW=3) and 2.6% from controls. The time in years from sample collection to diagnosis was 10.14 (mean), 9 (median, range: 1-23.5) and 14.33 (mean), 13.5 (median, range:1.8-31.4) in cases with detected paraprotein in the sample and those with no paraprotein detected, respectively. All cases diagnosed with MM or MW in the same year as the sample was collected were excluded from this analysis. The type of paraprotein detected was IgA in 33.4% of cases, IgG in 57% and IgM in 8.5%. *Conclusion.* This study indicates that MGUS precedes MM and WM in nearly half of the cases when analyzed with IF but only a quarter could be detected with PE. MGUS prevalence in the control subjects was in concordance with large population-based studies. The prevalence of IgA paraprotein in the MM cases with a prodromal MGUS phase was much higher than commonly reported in MGUS, reflecting the findings of other large studies that IgA MGUS has the highest risk of progression to malignant disease.

0738

PHASE I STUDY OF BORTEZOMIB AND 153SM-LEXIDRONAM COMBINATION FOR REFRACTORY AND RELAPSED MULTIPLE MYELOMA

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Background. Multiple myeloma (MM) is a highly radiosensitive B-cell malignancy and radiation therapy is an effective treatment for these patients. Recent preclinical studies have demonstrated that the bone-seeking radionuclide, Samarium Sm153 lexidronam (Sam) in combination with the proteasome inhibitor, bortezomib (Velcade [Vel]), can synergistically inhibit proliferation of myeloma cell lines *in vitro* and reduce MM growth in mice bearing murine MM without significant myelotoxicity. These results provide the basis for a new targeted therapeutic approach for refractory and relapsed MM patients that involve combining Vel with Sam to improve the anti-MM effects of these agents without increasing their toxicity. *Aims.* The primary objective of this dose escalation Phase I study is to determine safety and tolerability as well as the response rate as determined by the Blade criteria of Vel + Sam treatment for patients with relapsed or refractory MM. *Methods.* MM patients who had failed more than 2 prior treatments will be enrolled on this Phase I dose-escalation trial which involves six cohorts with three patients each. Previous treatment with Vel is allowed. Dose escalations in parallel arms are as attached. A complete treatment cycle is 8 weeks. Vel is given on days ,1 4, 8 and 11 followed by a 45-day rest period. Sam is administered only on day 3. The cycle is repeated on Day 57 if disease is stable or improved and platelets and neutrophils recover to better than or equal to Grade 1 toxicity (may be delayed for up to four weeks). Dose limiting toxicity (DLT) is defined as cycle 1 Grade 4 hematologic or Grade ≥3 non-hematologic toxicity.

Table 1. Outline of patient cohort.

	Arm 1			*Arm 2*	
	Sam	Vel		Sam	Vel
Cohort 1	0.25 mCi/kg	1.0 mg/m^2	Cohort 4	0.25 mCi/kg	1.3 mg/m^2
Cohort 2	0.5 mCi/kg	1.0 mg/m^2	Cohort 5	0.5 mCi/kg	1.3 mg/m^2
Cohort 3	1.0 mCi/kg	1.0 mg/m^2	Cohort 6	1.0 mCi/kg	1.3 mg/m^2

Results. Cohorts ,1 2 and 4 have been enrolled (3 patients per Cohort). Two patients (in Cohort 4) have shown responses, partial (n=1) and minor (n=1), and have received two cycles of treatment to date. Four patients progressed including one patient who showed a transient immunofixation+ complete response. Three patients in Cohort 2 have not completed their first cycle of therapy. No significant hematologic toxicities have been observed. Only one patient experienced transient fever, headache and vomiting. There have been no dose limiting toxicities to date. *Conclusions.* This dose escalation Phase I trial of the combination of Vel and Sam demonstrates responses in relapsed and refractory MM without significant toxicity and continues to enroll patients. Updated results from the trial will be presented at the meeting.

0739

ANTI-THYMOCYTE GLOBULIN INDUCES APOPTOSIS IN MYELOMA CELLS: A BASIS FOR MYELOMA SERO-THERAPY?

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Background. Monoclonal antibody based strategies have so far been unsuccessful in the treatment of myeloma. Polyclonal anti-thymocyte globulins (ATG) are used for *in vivo* T-cell depletion and have been reported to have cytotoxic activity against other cells including B-cells, dendritic cells and plasma cells. ATG is produced by immunization of rabbits or horses with thymocytes or T-lymphoblasts. *Aims.* We investigated the effect of ATG on myeloma cell lines and bone marrow samples from myeloma patients. We also studied the mechanisms behind ATG-induced myeloma cell death *Methods.* Apoptosis was detected by flow cytometry after staining with 7AAD and Annexin V. ZVAD-fmk was used for caspase inhibition, N-acetyl-L-cysteine (NAC) served as ROS scavenger. *Results.* We observed strong cytotoxic activity of ATG against myeloma cell lines and primary myeloma cells. Complement-dependent cytotoxicity (CDC) was observed in 5 of 5 myeloma cell lines (RPMI-8226, U266, KMS-12-BM, EJM and NCIH929) and bone marrow samples from 6 myeloma patients. In the absence of complement ATG still induced up to 50% apoptosis in 4 out of 5 myeloma cell lines and up to 80% apoptosis in all primary myeloma samples. Preincubation of myeloma cells with a general caspase inhibitor (ZVAD-fmk) abrogated ATG-induced apoptosis but had no effect on CDC. Preincubation with N-acetyl-L-cysteine (NAC), a ROS scavenger, blocked ATG-induced CDC but hat no effect on ATG-induced apoptosis. Absorption of ATG on primary T-cells completely removed anti-myeloma cytotoxicity. *Conclusions.* ATG induces complement mediated ROS-dependent lysis and caspase- dependent apoptosis in myeloma cells. This effect is probably due

to antibodies against epitopes also expressed by peripheral blood T-cells and not specific for myeloma cells or thymocytes and lymphoblasts used for the production of ATG.

0740

IMMUNOGLOBULIN-LIKE TRANSCRIPT 2 IS NOT DIFFERENTIALLY EXPRESSED IN MGUS AND MYELOMA, BUT APPEARS TO BE DOWNREGULATED AT AN EARLIER STAGE OF PLASMA CELL DISEASE

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Background. Immunoglobulin-like transcript 2 (ILT2) belongs to the Ig superfamily and has homology to the killer cell inhibitory receptors (KIRs). Like KIRs, ILT2 delivers an inhibitory signal upon interaction with MHC class I ligands. It is expressed on natural killer (NK) cells, which lyse transformed or virally infected cells that have lost or downregulated expression of self MHC class I molecules. ILT2 is also known to be expressed on monocytes, macrophages, dendritic cells, and (naïve) B lymphocytes. A differential expression of ILT2 was described for monoclonal gammopathy of undetermined significance (MGUS) and myeloma (Davies *et al*, 2003). Seven MGUS patients and 24 newly diagnosed myeloma patients were studied by gene expression profiling using Affymetrix GeneChip arrays. ILT2 was downregulated 8.26 fold in myeloma as compared to MGUS, being the most differentially expressed gene between these two subsets. However, as RNA from CD138+ cells was used in this analysis, a varying percentage of normal, non-malignant plasma cells will impact on the results, especially in MGUS cases. *Aims.* We aimed to delineate ILT2 expression in different plasma cell subsets (normal vs. malignant) in MGUS and myeloma and the eventual prognostic impact of a differential expression level. *Methods.* ILT2 expression was measured by flow cytometry using a PE-conjugated antibody (clone HP-F,1 Beckman Coulter, Inc.) in a series of 30 MGUS patients and 91 myeloma patients. Phenotypically normal and malignant plasma cells were defined by differential expression of markers CD38, CD45, CD19, CD56. Expression levels are given as mean fluorescence intensity (MFI) after correction for background staining. *Results.* ILT2 is not differentially expressed between MGUS (MFI median 112.0,1 range 13.45-274.42) and myeloma cells (MFI median 96.64, range 0.4-454.48). In contrast, MGUS/myeloma cells showed a lower expression of ILT2 as compared to phenotypically normal plasma cells in the majority of samples. An intraindividual comparison revealed a decrease in MFI in 70% of cases by a median of 63.3%, while in 30% of cases, there was an apparent upregulation of ILT2 in malignant cells (median increase in MFI of 30.0%). For myeloma, the variable level of ILT2 expression was confirmed by quantitative real time PCR in 26 cases. ILT2 levels did not vary with state of disease (newly diagnosed versus progressive disease). Also, we found no correlation of ILT2 expression with clinical parameters or prognosis in our series of myeloma patients, although, interestingly, 5 myeloma cell lines were completely ILT2 negative. *Summary/Conclusions.* ILT2 seems to be downregulated in the majority of cases at an early stage of plasma cell disease, i.e. upon transformation from a normal plasma cell to the MGUS/myeloma stage. The level of residual ILT2 expression in malignant plasma cells is neither correlated to the state of disease (MGUS versus newly diagnosed myeloma versus advanced disease), nor to prognosis of myeloma patients or other clinical parameters.

0741

A NEW MODEL PREDICTING AT LEAST A VERY GOOD PARTIAL RESPONSE IN PATIENTS WITH MULTIPLE MYELOMA (MM) AFTER 2 CYCLES OF BORTEZOMIB-BASED THERAPY

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Background. We recently reported that Velcade, Doxil, and Dexamethasone (VDD) is very active in both newly diagnosed and relapsed MM producing overall response rate up to 94% and complete and near complete response rate (CR/nCR) up to 33%. Despite these excellent response rates, the majority of patients do not achieve ≥90% reduction of disease (or ≥VGPR) which is considered a predictor of a longer remission and survival. Several recent studies showed that modification of therapy early in treatment in poor responders can improve quality of response. However, there are no established models to make early prediction of failure to achieve ≥VGPR. *Aims.* Using VDD as a model of Velcade-based therapy, we analyzed whether combination of normalization of free light chain (FLC) ratio and reduction of serum M-protein can be used as an early predictor of ≥ VGPR response in MM. *Methods.* Thirty-six patients who were enrolled on IRB approved phase II trials with VDD in newly diagnosed and relapsed MM were eligible for analysis. Ultimate responses were assigned using the EBMT criteria after 6 cycles or if after 2 cycles of chemotherapy the patient exhibited at least VGPR. Patients received a score of 1 if they had either normalization of FLC ratio (provided there was a reduction in involved FLC by ≥90% from the baseline) or a reduction in their pre-treatment monoclonal protein by ≥≥ 90% after 2 cycles. If both criteria were met a score of 2 was assigned. If neither were met a score of 0 was given. The Fisher's exact test was used to compare the score in patients exhibiting a ≥ VGPR to those with ≤ VGPR. *Results.* Of the 22 evaluable patients with VDD in relapsed disease, 7 exhibited a ≥ VGPR, with 15 ≤VGPR. Of the 14 evaluable patients with VDD as first line, 7 demonstrated a ≥ VGPR, and 7 ≤ VGPR. All patients with ≥ VGPR except for one in relapsed VDD protocol, had a score of 1 or 2 compared to those with ≤ VGPR, who all had a score of 0 (p<0.0001). *Summary/Conclusions.* In both relapsed and first line therapy, a normalization of FLC ratio or reduction of serum monoclonal protein by ≥90% after 2 cycles of chemotherapy accurately predicts at least VGPR response to chemotherapy. In future trials with Velcade-based regimens, early modification of therapy could be planned if a patient does not demonstrate a normalization of FLC or ≥90% reduction of serum monoclonal protein after the initial 2 cycles.

0742

RARE OCCURRENCE OF T(4;14) AND P53 DELETION BUT HIGH INCIDENCE OF OTHER MYELOMA HIGH-RISK FEATURES (+1Q, +9Q, AND 13Q-) IN MGUS ANALYSIS USING FISH AND DNA PROBES FOR THE DETECTION OF GENOMIC ABNORMALITIES INVOLVING 10 CHROMOSOMAL LOCI

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Background. The biology of the transition of monoclonal gammopathy of undetermined significance (MGUS) to multiple myeloma (MM) is poorly understood but one can assume that this process is closely linked to the accumulation of clonal aberrations in the neoplastic cell. In MM, some chromosomal abnormalities (13q-, 17p-, +9q, t[4;14] and amplification of CKS1B at 1q21.2) are of prognostic relevance as they are associated with shorter survival. *Methods.* So far, bone marrow specimens from 48 patients diagnosed with MGUS at our institution were analyzed by FISH and a DNA probe set originally designed for the evaluation of MM. The probe set comprises probes mapping to chromosome bands 1p22, 1q21.2, 6q2,1 8p1,1 9q34, 11q25, 13q14, 17p13, 22q1,1 and 14q32 (including probes for the detection of t[11;14] and t[4;14]). Purification of PC by immunomagnetic separation (CD138) was performed in 38 of 45 cases. *Results.* The most frequent chromosomal imbalances in the entire cohort were: +9q (13/44-29%), t(11;14) (8/28-28%), +11q (10/48-21%), 13q- (10/48-21%), and +1q (9/46-19%). No p53 deletion was detectable in 48 patients. Chromosomal extra copies were significantly more prevalent in patients lacking an IgH translocation (p=0.047). In 7 patients for that follow-up samples are available, there was no evidence for clonal evolution by means of occurrence of additional abnormalities or increasing size of aberrant clones (analysis ongoing). To date, only one patient with +6q, +9q, +11q, and t(11;14) progressed to MM. *Conclusions.* The vast majority of patients with MGUS exhibit chromosomal abnormalities. 13q-, +1q, and +9q ' markers of an inferior outcome in MM - are frequently found in MGUS while t(4;14) seems to be rare. No p53 deletion was found in the present series. Chromosomal extra copies were significantly more prevalent in patients lacking a 14q32 translocation.

0743

SCREENING OF JAK2 V617F MUTATION IN MULTIPLE MYELOMA

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Background. JAKs tyrosine kinases are important mediator of cellular signals between cytokines, receptors and effector proteins. They have 7 structural domains 'JAK homology regions' (JH1-JH7) in particular JH1 and JH2: JH1 has kinase activity, while JH2 has a negative-regulatory function on JH1. Recently a somatic mutation in exon 12 of JAK2 has been described in myeloproliferative diseases Philadelphia Chromosome negative as PV, ET and IMF and more recently this mutation has been investigated also in AML, MDS, aCML (BCR-ABL negative), ALL and CLL. JAK2 mutation was identified in a subset of CMML/ aCML, and

MDS but not in B-ALL, T- ALL or CLL. This mutation results in a substitution of valine for phenylalanine at position 617 (V617F) in the JH2 domain and leads to constitutive tyrosine phosphorilation and cytokine hypersentivity. Alterations of JAK/STAT signalling molecules with a constitutive activation of STATs have been reported for several lymphoma as in PMBL and cHL and screening for the presence of the mutation has revealed the absence of JAK2 V617F from all cell lines, PMBLs and HLs. *Aims.* We investigated the presence of JAK2 V617F mutation in Multiple Myeloma, a B-cell neoplastic disease characterized by bone marrow infiltration from malignant plasma cells which secrete monoclonal immunoglobulin fragments. Although several parameters such as β2-microglobulin, serum creatinine, hemoglobin, calcium levels or cytogenetics abnormalities have been taken account as predictive factors of the outcome of patients affected by MM, the molecular features of this disease remain still unclear. Cytokines of interleukin 6 family (IL6) which activate the signals transducers gp130, are major survival and growth factors for MM cells. The signal transduction of gp130 involves JAK,1 JAK2 and TyK2 and then the downstream effectors comprising the signal transducer and activator of transcription 3 (STAT3) and mitogen-activated proteine kinase (MAPK) pathways. Some authors found that an inhibitor of JAK2, AG490, suppressed cell proliferation and induced apoptosis in IL-6-dependent MM cell lines. JAK2 kinase activity, ERK2 and STAT3 phosphorylation were inhibited. *Methods.* To detect the JAK2 V617F mutation we performed allele-specific PCR using genomic DNA from peripheral blood samples of 93 consecutive patients affected by MM. All samples were collected after informed consent from 2002 and were mostly taken at diagnosis. *Results.* Patients'charactheristics were: median age at diagnosis 66 years (range 35-88), M/F 51/42, Immunoglobulin (Ig)G 52/93 (56%), IgA 28/93 (30.1%), micromolecular 9/93 (9.7%), IgD 2/93 (2.1%), IgM 1/93 (1.1%), Stage I 10/93 (10.8%), stage IIA 35/93 (38%), stage IIB 2/93 (2.1%), stage IIIA 44/93 (47%), stage IIIB 2/93 (2.1%). All 93 MM samples analyzed were wild type for the JAK2 V617F mutation and presented the only internal control on electrophoresis agarose gel (364 bp) and not the 203 bp product, indicative of JAK2 mutation V617F. *Conclusions.* Thus the mutation of JAK2 V617F is absent in MM and we can suggest that JAK2 mutation V617F does not play a role in the pathogenesis of MM. Given the importance of Jak2 activation in MM, a comprehensive mutational screening of its coding exons is warranted.

0744

OZONE THERAPY IN THE TREATMENT OF OSTEONECROSIS OF THE JAWS IN MULTIPLE MYELOMA PATIENTS

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Background. Bisphosphonate (Pamidronate and/or Zoledronate) therapy is commonly prescribed for the prevention and cure of pathological fractures in Multiple Myeloma (MM) patients. Among the several properties of this drug its osteoclastic inhibition results in reduction of bone resorption. Since 2003 a possible association between bisphosphonate use and the appearance of osteonecrosis of the jaws, especially in long-treated patients undergoing invasive oral procedures, has been reported. Histologic examinations show these lesions to be the result of an avascular necrosis of the bone, due, in first ipothesis, to bisphosphonates osteoclastic and angiogenetic inhibition, which impairs healing and exposes to infections by oral bacteria. *Aims.* The limited benefits recorded in patients suffering from this severe complication with the treatment options so far utilized, i.e. antibiotics with or without surgery and hyperbaric oxygen therapy, prompted us to explore the use of ozone therapy, whose antimicrobic action and neoangiogenetic properties have been shown capable of arresting the progression of dental lesions. *Methods.* Since 1998 up today, in our Institute we treated 311 Multiple Myeloma patients with Pamidronate 90 mg i.v. and/or Zoledronate 4 mg i.v. monthly. Twenty-two (7%) patients referred toothache, impaired healing after teeth extractions, dental abscesses and bone exposure, bisphosphonates were withdrawn and antibiotics administered. Considering the limited benefits in this subset of patients with the standard therapy we decided to follow a 15 day treatment protocol including antibiotics (amoxicillin- clavulanic acid 2 gr/daily plus metronidazole 1 gr/daily), surgery (from simple curettage to bone reseption) and ozone therapy (administered previous, during and after surgery). Response: Among the 22 patients with dental abscesses or jawbone exposition, 12 patients are evaluable for response because they completed the program. Ten were symptomatic MM treated with chemotherapy and 2 smoldering myelomas, 9 were women and 3 men with a median age of 72 years (range 58-79). Eight patients were IgG, 3 IgA and 1 light chain. Seven patients had received Zoledronate and 5 Pamidronate followed by Zoledronate for a median time of 19 months (range 6-63). One patient had a wide bone exposition with oro-sinusal fistula, 10 had difficult healing after teeth extractions or oral cleaning and 1 developed ONJ spontaneously. All patients presented pain, secretion and halitosis that were evaluated by questionnaires administered before and after the treatment. Nine patients (75%) have witnessed complete resolution of the problem with a total riepithelialization of the lesions and 4 (25%) an objective improvement. *Conclusion.* These results demonstrate that the association of ozone therapy with antibiotics and surgery is an effective treatment for avascular necrosis of the bone.

0745

EFFECTS AND MOLECULAR MECHANISM OF LENALIDOMIDE ON FGFR SIGNALING IN ENDOTHELIAL CELLS AND FGFR3+ MULTIPLE MYELOMA CELL LINES

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Background. Lenalidomide (Revlimid®) has recently been approved for the treatment of a subset of myelodysplastic syndromes, and is being evaluated as treatment for a broad range of other hematology and oncology conditions, including multiple myeloma (MM), chronic lymphocytic leukemia and solid tumor cancers. Data from Phase II and Phase III studies of lenalidomide plus dexamethasone in MM patients show significant benefits of combination therapy versus dexamethasone alone, in terms of median time to disease progression and overall survival. A subset of MM patients (10-20%) are known to have a t(4;14) translocation that results in the overexpression of oncogenes FGFR3 and MMSET/WHSC1. FGFR3 is not normally expressed in B cells but is overexpressed and sometimes has a constitutively activating mutation in multiple myeloma cells with t(4;14). FGFR3 is an oncogenic tyrosine receptor kinase that is activated by the pro-angiogenic growth factors aFGF and bFGF. FGFR3 signals activate the MAPK pathway via Shc and Grb2 scaffolding complexes in FGFR3+ MM cells, while FGFR signals activate the Akt pathway in ECs. We hypothesized that inhibition of FGFR3 signaling may be one of the mechanisms of lenalidomide action. *Aims.* The present study examines the effect of lenalidomide on FGF-induced signals in endothelial cells and t(4;14) MM cells. *Methods.* EC migration assay. HUVECs (5×10^4 cells/insert) were assayed for migration in response to bFGF (0.1 ng/mL) using the BD BiocoatTM Angiogenesis System using 3 mm pore size fibronectin coated filters. Cells were allowed to migrate±lenalidomide for 22±1 h, labeled post migration with Calcein AM (Molecular Probes) and measured by fluorescence of migrated cells using a microplate fluorescence reader (Bio-Tek). Cell proliferation assay. Cells were incubated in 96-well cell culture plates with compounds for 72 hours and assayed by 3H-thymidine incorporation. IC50s were calculated by nonlinear regression analyses with GraphPad Prism. Immunoblot. Cells were treated with DMSO or lenalidomide then stimulated with aFGF for 10 minutes. Lysates were probed with phospho-Shc, phospho-cRaf, phospho-Ras, phospho-MEK1/2 or phospho-Erk1/2 Abs (Santa Cruz) and analyzed on a Storm 860 Imager using ImageQuant software (Molecular Dynamics). FGFR3 enzymatic assay. Lenalidomide was tested using the KinaseProfiler Assay (Upstate Biotechnology). Luciferase assay. Cells were transfected AP1-luciferase (Stratagene). Cells were pre-treated with 1 μM lenalidomide or DMSO for 1 hour then stimulated with aFGF overnight. Luciferase activity was assayed using luciferase substrate (Promega) and measured using a luminometer (Turner Designs). *Results.* Lenalidomide was found to inhibit bFGF-induced responses in endothelial cells, reducing cell migration through fibronectin-coated membranes, and suppressing phosphorylation of the scaffolding protein Gab1 and the serine-threonine kinase Akt. However, in aFGF-stimulated FGFR3+ LP-1 cells (t(4;14), FGFR3 F384L), which are sensitive to lenalidomide (IC50=0.78 μM), lenalidomide has no effect on the phosphorylation of Shc, cRaf, Ras, MEK1/2, or Erk1/2. Nor does lenalidomide have an effect on FGFR3 activity itself. Lenalidomide does inhibit aFGF-stimulated AP-1 transcriptional activation. *Conclusions.* These data suggest that lenalidomide's effects in endothelial cells and multiple myeloma cells involve inhibiting FGF signaling, albeit by different mechanisms.

0746

TUMOR ANGIOGENESIS AND SENSITIVITY TO THE IL-6 IN MULTIPLE MYELOMA: EXPRESSION OF THE MICROVESSEL DENSITY AND GP-130 INTERLEUKIN-6 TRANSDUCER WITHIN THE BONE MARROW COMPARTMENT

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The functional interplay between the myeloma cells and the surrounding microenvironment within the bone marrow (BM) includes increased activity of endothelial cells resulting in neovascularisation, and enhanced sensitivity to the IL-6 as a main growth factor in multiple myeloma (MM). This cytokine, as a member of gp130 family, binds on the surface of myeloma cells to the IL-6 receptor α chain that associates with the gp130 transducer chain (CD130), providing the proliferation signal to the tumor cells. The aim of study was to investigate the correlation between expression of BM angiogenesis estimated as microvessel density (MVD), and expression of the transmembrane signal transducer, gp130, in the bone marrow of MM patients (pts). The study included 60 newly diagnosed MM pts (33 male and 27 female pts, mean age 60 years, range 35-75). According to the clinical stage (CS, Salmon&Durie), distribution of MM pts was as follows: I 8pts, II 22pts, III 30pts. There were 35pts with IgG monoclonal (m) protein, 12pts with IgA, and 12pts with secretion of kappa/lambda chain. None secretory MM was diagnosed in 1pts. All pts were treated with standard chemotherapy regimens. BM vessels were visualized by immunohistochemical staining for CD34 (BI-3C5, Santa Cruz Biotechnology, USA) on slides of formalin-fixed, paraffin-embedded BM biopsies. MVD was calculated by the number of vessels per 400x high-power microscopy field in the area of the most dense vascularization. All samples were further analyzed for the immunohistochemical expression of the gp130 (AN-H2, Santa Cruz Biotechnology, USA) which showed cytoplasmic and membrane localization. The intensity of these stainings was graded as weak (0-30% myeloma cells), moderate (31-60% myeloma cells), and strong (>60% myeloma cells). Control specimens were obtained from pts without hematological malignancy. According to the CS of myeloma, positive correlation was found between MVD and expression of GP130 in myeloma cells. The expression of MVD was significantly higher in MM pts in III CS than in pts in I CS of myeloma (15 vs. 7,5/ ×400 field, $p< 0,001$). Similarly, significantly higher expression of gp130 was found in pts in III CS of myeloma comparing to the MM pts in I CS (32 vs.15%, $p<0,05$). These findings of increased angiogenesis in correlation with high IL-6 sensitivity found in IIICS of myeloma pointed out significantly shorter survival of those pts (26 vs. 43,5 m, log rank, $p<0,05$). In conclusion, strong activity of angiogenesis in myeloma, combined with high IL-6 sensitivity by immunohistochemical expression of gp130 represents possible predictive factors of poor prognosis.

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CORRELATION BETWEEN THE CYTOGENETIC FINDINGS AND THE PROGNOSTIC FACTORS IN THE GROUP OF PATIENTS FROM THE CMG 2002 CLINICAL STUDY

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Background. Cytogenetic abnormalities in multiple myeloma (MM) are one of the most important independent prognostic factors. *Aims.* To determine the correlation between the aberration of the chromosome 13 (detected by molecular cytogenetic methods) and the prognostic factors in the pilot group of patients from the CMG 2002 clinical study (only data from one clinical centre covers 1/4 of patients) using three various of cut off levels (9%, 20%, 80%). *Methods.* Interphase fluorescence *in situ* hybridization (I-FISH) and fluorescence *in situ* hybridization and cytoplasm immunoglobulin staining (cIg-FISH) were used to detect the aberration of the chromosome 13. Cytogenetic abnormalities were found in 65 newly diagnosed patients with MM, the median of follow up was 22.8 month. *Results.* The aberration was found in 40% (26/65) patients (cut off levels 9%, 20%) and in 21.5% (14/65) patients (cut off level 80%). We have correlated standard prognostic factors (MIG, LDH, β2M, Hb, platelet count, albumin), event free survival (EFS), and overall survival (OS) with the occurrence of the aberration of chromosome 13 detected by I-FISH on bone marrow slides and cIg-FISH. Higher MIG and lower albumin concentrations and platelet counts were detected in patients with aberration of chromosome 13 (cut off levels 9%, 20%), similar results were obtained for cut off level 80%. No prognostic significance was found between aberration of chromosome 13 and the worst prognostic feature (EFS shorter than one year) in all cut off levels for aberration of chromosome 13. *Summary/Conclusion.* We have analysed the data from homogenous group of patients undergoing autologous transplantation in the CMG trial of Czech Myeloma Group. We have correlated standard prognostic factors (MIG, LDH, β2M, Hb, platelet count, albumin), EFS, and OS with the occurrence of the aberration of chromosome 13 using three variants of cut off levels (9%, 20%, 80%). Higher MIG and lower albumin concentrations and platelet counts (for cut off level 80%) were detected in patients with aberration of chromosome 13 (cut off levels 9%, 20%). This analysis will be extended for all centres of CMG 2002.

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LOW-DOSE THALIDOMIDE AS MAINTENANCE THERAPY FOLLOWING SINGLE OR TANDEM AUTOTRANSPLANT IN ADVANCED MULTIPLE MYELOMA IMPROVES OVERALL RESPONSE WITH MILD TOXICITY

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Background. Thalidomide has been introduced few years ago in the treatment of MM. At present is part of many clinical trials, especially as front line therapy in combination with desamethasone or chemotherapy. Although the activity of Thalidomide as monotherapy is widely accepted in relapsed or refractory MM, its role as maintenance therapy following autotransplant is still under investigation. The drug is effective but the toxicity, i.e the DVT, remains one of the main reasons of concern for many investigators, so that the schedule, dose and anti-thrombotic prophylaxis are still matter of debate. *Methods.* In 1999 we started a trial with conventional chemotherapy (3 cycles of VAD), followed by high-dose cyclophosphamide (7 g/m^2 i.v.) and peripheral stem cells (PBSC) harvest, followed by single or tandem autotransplant with melphalan (200 mg/m^2 i.v.), in patients affected by advanced MM (stage II-III Salmon-Durie). Thalidomide 100 mg a day was then given as maintenance to all patients regardless the type of response, and discontinued at the time of relapse or progression, or for toxicity. No anti-thrombotic prophylaxis has been administered. *Patient characteristics*. Between January 1999 and June 2005, 75 consecutive MM patients were enrolled. Seventy patients, median age 55 (range 46-66 years), M/F 43/27, are valuable. All these patients completed chemotherapy without major problems, and no toxic deaths occurred: 10/70 patients were in complete remission (CR) at the time of PBSC transplant, 34/70 reached CR after transplant (60/70 cases underwent tandem transplant), so that after chemotherapy 44/70 (62%) were in CR, defined as bone marrow plasmacytosis below 5% and absence of serum and urine paraprotein. Thalidomide was started when possible within 6 months following transplant: 21/70 patients could not be treated because of different reasons: progression of disease (6 cases), psychological problems (3), performance status <70% (2), neurological problems (2), refusal (1). Three cases were followed in other Institutions. Only 4 patients discontinued the drug in few weeks because of mild neurological toxicity (WHO < 2). The remaining 49 patients (70%) continued the drug until relapse or progression, for a median time of 24 months after transplant. *Results.* The CR rate after PBSC transplant was 69% in the group treated with thalidomide and 52% in the remaining patients. With a median follow-up of 38 months we compared the number of relapses/progressions, the time to relapse/progression, the disease free survival (DFS) and the overall survival (OS) in the two groups of patients. *Toxicity.* Most patients reported peripheral neuropathy, somnolence and constipation: when severe, a temporary adjustment of drug dose was able to control these symptoms. Despite the absence of anti-thrombotic prophylaxis, no DVT were observed. *Conclusions.* Low-dose Thalidomide following single or tandem autotransplant appears to be a safe and feasible maintenance treatment improving overall response rate without severe side effects. No anti-thrombotic prophylaxis is needed.

Table 1. In results, after'in the two groups of patients.

	rel/progr (%)	*time rel/prog*	*4 ys OS%*	*4 ys DFS % (p<0.08)*
with thal	23/49 (46%)	31 mo	76	45
w/o thal	16/21 (76%)	24 mo	35	35

0749

PEGYLATED LIPOSOMAL DOXORUBICIN, MELPHALAN AND PREDNISONE THERAPY FOR ELDERLY PATIENTS WITH MULTIPLE MYELOMA

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Background. Melphalan & Prednisone (MP) is considered as the standard therapy for Multiple Myeloma (MM) patients not eligible for high dose therapy, but the addition of new drugs could result in better resultsd. *Aims.* Here we report the results of a phase I-II study to evaluate the feasibility and efficacy of the association of PLD to the conventional MP regimen during the first 6 cycles of the front-line therapy for untreated MM patients older than 70. *Patients and Methods.* Thirty patients were included in the study with a median age of 77 years (71-84) and a M/F ratio of 17/13 in a phase I/II study to determine the best dose of PLD and the rewsponse rate. *Results.* The phase I of the study demonstrated that the maximum tolerable dose of PLD in this setting was 30 mg/m^2, so it was the final dose evaluated in the study. 29 patients were valuable for response, which was: complete in 4 (14%), partial in 15 (52%), minor/no changes in 7 (24%) and progressive in 3 (10%). The median progression free survival (PFS) was 24 months. The median overall survival (OS) has not been reached yet, with a 3-year probability for OS and PFS of 52% and 37%, respectively. Hematological toxicity was frequent but usually weak/moderate (grades 1 & 2 of the WHO scale) and it was resolved only with dose delays. Infection was a relatively frequent event (30% of patients), but only in 4 cases it was of grade 3. No cases of palmar-plantar erythrodysesthesia were observed. *Conclusions.* Elderly MM patients can benefit from other more intensive therapeutic alternatives than MP as the addition of pegylated liposomal doxorubicin to this conventional regimen.

0750

INCREASED INHIBITORY, CD158A, RECEPTOR EXPRESSION ON CD16+NK CELLS AND IMPAIRED NK CELL CYTOTOXICITY IN ADVANCED MYELOMA PATIENTS

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Background. The inability of the immune system to recognize and kill malignant plasma cells in patients with multiple myeloma (MM) has been attributed in part to the ineffective activation of natural killer (NK) cells. The activity of NK cells is regulated by opposing, activating and inhibitory, receptors and their balance, as well as the influence of cytokines, determines NK cell cytotoxicity. *Aim.*The aim of this study was to evaluate NK cell activity in the light of the expression of novel NK cell activating and inhibitory receptors in myeloma patients. *Methods.* In this study in 20 MM patients in clinical stage III and IV, prior to therapy, and in 15 controls NK cell activity, percent of innate cell subsets, expression of activating (CD161) and inhibitory (CD158a, CD158b) receptors on freshly isolated PBL and CD16+NK cells were evaluated using 51-chromium release assay and direct immunofluorescence by Flow cytometry. *Results.* We show significant impairment of NK cell activity without any change in the percent of innate immunity subsets (CD16+NK, NKT and CTL$\gamma\delta$). There is a significant increase in the CD16dim NK cell subset in PBL in MM patients compared to controls. There is no decrease in CD161 activating receptor (MFI of CD161 on CD16bright is significantly higher), or increase in CD158b inhibitory receptor, expression on fresh PBL or CD16+NK cells, while, there was a significant increase in the inhibitory, CD158a, receptor expression on CD16+NK cells in MM patients. *Conclusion.* We give novel results for advanced multiple myeloma patients that show that an increase in the immature CD16dim NK cell subset and an increase in the expression of KIR, CD158a inhibitory receptor, on CD16+NK cells has an adverse effect and is associated with impaired NK cell cytotoxicity. Aside from this, these findings may have implications in developing therapeutic approaches in multiple myeloma which use recombinant NK receptor ligands that aid in targeting NK cells to tumor cells.