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## ABSTRACTS

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## **APLASTIC ANEMIA AND ACUTE LEUKEMIA**

### **C001**

#### **ACUTE LEUKAEMIA IMMUNOPHENOTYPING IN BONE MARROW ROUTINE SECTIONS**

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Immunohistochemistry of acute leukaemias in bone marrow paraffin sections is commonly thought to be useless because of the poor preservation of many lineage-related markers. However, the recent development of monoclonal antibodies against fixative-resistant epitopes and of new antigen retrieval techniques has expanded the possibility of accurately testing routine samples. To assess the relevance of paraffin section phenotyping in lineage determination, 110 samples from acute leukaemia patients were studied by specific antibodies against CD1a, CD3, CD15, CD20, CD34, CD68, CD79a, TdT, myeloperoxidase, glycophorin A and factor VIII-related antigen. 59 acute myeloid leukaemias, 39 precursor B-cell acute lymphoblastic leukaemias (B-ALLs), 7 T-ALLs and 5 mixed precursor B-cell/myeloid acute leukaemias were included. The combination of the markers employed allowed the identification of the cell lineage (myeloid, lymphoid or mixed) in all the cases and, in some instances, of phenotypic profiles characteristic of distinct acute leukaemia subtypes. According to the results obtained, bone marrow biopsy may be regarded as a reliable tool for acute leukaemia diagnosis; this observation is of practical relevance especially for the classification of cases which lack circulating blasts in the peripheral blood or showing dry tap at bone marrow aspiration.

### **C002**

#### **CYTOGENETIC STUDY OF 256 ADULT PATIENTS WITH ACUTE LYMPHOBLASTIC LEUKEMIA (ALL) AT DIAGNOSIS**

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A prospective conventional cytogenetic (CC) investigation on 256 adult patients with ALL entered into the GIMEMA 0496 trial has so far been carried out on bone marrow samples taken at diagnosis and centralized, by overnight dispatch, in Rome. A cytogenetic result was obtained on 163/256 (64%) of the cases analyzed. The patients were classified into cytogenetic subgroups by structural abnormalities and, subordinately, by ploidy, to highlight structural changes of prognostic relevance. A karyotype was considered as normal if an abnormal clone was not identified on the analysis of at least 10 cells. Abnormal karyotypes were detected in 101/163 (62%) cases successfully investigated. The following cytogenetic subgroups have been identified: t(9;22)(q34;q11) (36 cases, 22%), t(4;11)(q21;q23) (10 cases, 6%), del(6q) (10 cases, 6%), del(7q) (5 cases, 3%),  $\leq$  than two structural or numerical changes (23 cases, 14%),  $\geq$  than three structural or numerical changes (17 cases, 10%). When classified by ploidy the 163 cases were distributed as follows: hypodiploid (4 cases, 2.5%), normal (62 cases, 38.5%), pseudodiploid (73 cases, 45%), low hyperdiploid (16 cases, 10%), high hyperdiploid (6 cases, 4%), hypotriploid (2 cases, 1%). The interim correlation with the clinical outcome has been done on 124/256 patients with at least three months follow-up from diagnosis. Eighty-five of these patients were cytogenetically categorized by structural changes. As expected, patients with the Ph chromosome and t(4;11) had a significantly worse outcome, with percentages of relapse of 63% and 57% ( $p = 0.027$ ), respectively. A high relapse rate (67%) was observed in patients with del(6q), a subgroup considered to have a favorable prognosis. Overall, patients with an apparently normal karyotype did significantly better than those with an abnormal karyotype (85% vs 48% of patients in persistent com-

plete remission,  $p=0.005$ ). Also of interest was the apparently poor prognosis (46% of relapses) of cases classified as not evaluable because of less than 10 apparently normal metaphases. This study highlights the feasibility of a centralized cytogenetic investigation and re-emphasizes the prognostic role of cytogenetics for adult ALL, providing a rationale for the development of stratified treatment approaches based on diagnostic cytogenetics.

### CO03

#### PROGNOSTIC SIGNIFICANCE OF CD56 EXPRESSION IN ACUTE MYELOID LEUKEMIA PATIENTS

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CD56 (NCAM) is a 220 kd glycoprotein predominantly expressed on human natural killer cells. It has been reported that CD56 antigen is expressed also in acute myeloid leukemia cells (AML), generally associated with monocytic morphology and extramedullary involvement. Recently, it has been shown that in t(8;21) AML the expression of CD56 antigen identifies patients with poor prognosis. On the basis of these recent findings, we evaluated surface CD56 expression of leukemic cells in 113 newly diagnosed AML patients and results were correlated with other prognostic factors and clinical outcome. CD56 antigen was present in 29/113 cases (26%) with a percentage of positive cells ranging from 11% to 97% (median 43). No correlation was found with FAB cytotype or chromosomal abnormalities. CD34 antigen was coexpressed in 16/29 (55%) of CD56 positive cases. Following standard intensive chemotherapy a significant reduction of complete remission rate was recorded, in fact only 37% of CD56 positive evaluable patients achieved complete remission, while the remaining patients were resistant to the treatment or experienced only a partial response. In conclusion, since it appears that CD56 expression on myeloid blasts identifies patients with poor clinical outcome our results sug-

gest that CD56 analysis may be useful to identify AML patients which need a more aggressive therapeutic approach.

### CO04

#### C-KIT MUTATIONS ARE A RELATIVELY FREQUENT EVENT IN PROGRESSION OF AML M2

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Detection of a novel activating c-kit mutation (Asp816Tyr) in a patient with AML M2 with mast cell involvement and karyotype 47, t(8;21) +4 (<sup>1,2</sup>) raises two main questions: i) the role of c-kit mutation in the observed leukemia, ii) the incidence of c-kit mutations in AML M2. Study of the dosage of the mutated c-kit allele in leukemic blasts carrying trisomy 4, the chromosome where the c-kit gene is located, demonstrated that tris 4 leads to duplication of the mutated allele in blasts carrying the primary t(8;21) rearrangement. Trisomy 4 thus appears associated with leukemia progression. To address the second query AML M2 with the typical antigenic combination (CD34<sup>+</sup>, CD117<sup>+</sup>, CD13<sup>+</sup> and CD33<sup>+</sup>) and t(8;21), with or without additional numerical chromosomal changes, were selected and screened for known mutations at codon 816, such as the observed Asp816Tyr and the Asp816Val, which is prevalent in mastocytosis. Digestion of amplified DNA using restriction enzymes suitable to distinguish between the normal and the mutated allele allowed to identify the Asp816Val mutation in three out of nine cases investigated. Significantly, the first case has a t(8;21) +4 karyotype, the second has t(8;21) +13, while the third carries a t(2;8;21). These findings indicate that activating c-kit mutations are not a rare event in AML M2, a CD117<sup>+</sup> FAB subtype, likely prone to c-kit mutation and responsive to mutation effects. The prognostic meaning of c-kit mutation as regards leukemia evolution should be evaluated by accurate fol-

low-up of positive patients as compared to negative patients carrying t(8;21).

<sup>1</sup> Beghini A. et al. *BMCD* 24(2): 262, 1998

<sup>2</sup> Beghini A. et al. *Blood* 92(2): 701, 1998

## C005

### TEL/AML1 REARRANGED CHILDHOOD ALL

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The t(12;21)(p13;q22) translocation, cryptic at the cytogenetic level (0,05%) has resulted to be the most frequent genetic abnormality when investigated by molecular analysis for the TEL/AML1 hybrid transcript (20-25%). This translocation has been associated with age 1-10 years, B precursor lineage involvement (frequently with myeloid antigens coexpression), and excellent EFS(90% at 5 years). In order to investigate the prognostic significance of TEL/AML1 presence at diagnosis of ALL, we have studied the preliminary results of the prospective study on childhood ALLs consecutively diagnosed at our Institution between 1/5/95 and 31/12/98, and uniformly treated with the multicentric AIEOP protocol. The TEL/AML1 transcript has been investigated by RT-PCR on the diagnostic BM samples, and detected in 18/75 patients (24%). All rearranged patients had B-precursor involvement, myeloid markers in 13/18 and age 1-7 years (median: 4). 73/75 patients went in CR: 18/18 in the t(12;21) positive group and 55/57 in the negative group. During follow-up (4-48 months, median 20) 9 patients experienced a BM relapse: 3/18 (16,6%) in the rearranged group, 6/55 (10,9%) in the negative group. The 3 relapses observed in the TEL/AML1 patients have occurred in off-therapy (1st CR length: 28,28,40 months), while in the negative group 5/6 relapses have occurred during treatment (1st CR length 10,10,12,13,15 months) and only in 1 case in off-therapy (30 months). At present, 3/3 TEL/AML1 positive relapsed children are alive in 2nd CR (follow-up 5-8 months), contrasting with only 2/6 alive in the TEL/AML1 negative series. Our data show that TEL/AML1 rearranged patients, though

bearing the typical favourable biological and clinical findings and good therapeutic response, may relapse with an unexpected and relatively high rate when compared to the t(12;21) negative series. However in the TEL/AML1 relapsed patients 1st CR appears to last longer (of at least 12 months) than in the negative group. Our data, though will undoubtedly benefit from additional follow-up, appear to argue against the "excellent" prognostic significance of TEL/AML1 rearrangement and warn against the proposal of considering a less intensive treatment in these patients.

## C006

### MULTI DRUG RESISTANCE IN ACUTE PROMYELOCYTIC LEUKEMIA (APL): A LOW PGP, LRP AND MRP EXPRESSION MAY CONTRIBUTE TO THE HIGH SENSITIVITY TO CHEMOTHERAPY OF APL

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Acute Promyelocytic Leukemia (APL), is a biologically and clinically well defined subtype of Acute Myelocytic Leukemia that from a clinical perspective shows an high sensitivity to Anthracyclines. We hypothesized that APL blasts may have low levels of drug transporter proteins. Therefore the expression of the P-Glycoprotein (PGP), of the Multidrug Resistance associated Protein (MRP), of the Lung Resistance related Protein (LRP) and the Intracellular Daunorubicin Accumulation (IDA) were evaluated in consecutive 23 APL cases diagnosed at the Division of Haematology of the University Hospital of Udine based on morphology, detection of the t(15;17) and/or chimeric fusion transcript PML/RAR $\alpha$  between february 1990 and december 1998. PGP, LRP and MRP expressions were evaluated by flow cytometry using the MRK-16, the LRP56 and the MRPm6 monoclonal antibodies. The IDA was evaluated by flow cytometry after a 2 hour incubation in 1000 ng/ml Daunorubicin. A PGP, LRP, and MRP overexpression was defined for MRK-16, LRP56 and MRPm6 Mean Fluorescence Index higher than 6, 5 and 3 respectively that

is for PGP, LRP or MRP expression higher than the ones observed in non MDR cell lines and in normal mononuclear cells taken from bone marrow of healthy donors. A defect of IDA was defined by Normalized Mean Fluorescence Index lower than 300 that is for anthracycline levels lower than the ones observed in normal mononuclear cells taken from bone marrow of healthy donors. At onset, the median MRK-16 MFI was 3.6 (range 2.7 - 5.6), the median LRP56 MFI was 2.4 (range 1.1 - 6.0) and the median MRPm6 MFI was 1.3 (range 1.0-2.3). No cases were defined as PGP or MRP overexpressing. An LRP overexpression was observed in 1/23 cases. The mean IDA was  $402 \pm 101$ . A defect of IDA was found in 2/23 cases. During the period of the study, 6 patients relapsed and the MDR phenotype has been resetted. Again at the first relapse, only one case overexpressed LRP and had a defect of blast cells' IDA. This study suggests that a low PGP, LRP and MRP expression and the absence of defects of anthracycline Accumulations may contribute in providing the biological basis for the high sensitivity to chemotherapy of APL.

## CO07

### ACUTE PROMYELOCYTIC LEUKEMIA (sAPL) FOLLOWING A PREVIOUS MALIGNANCY. EXPERIENCE OF GIMEMA

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**Objective:** To evaluate the clinical and laboratory characteristics of adult patients affected by sAPL developed after a previous malignancy (PM). **Design:** Retrospective study, conducted over a fourteen-years period (1984-1997). **Setting:** 62 hematology division in tertiary care or university hospital. **Results:** During the study period were observed 51 sAPL (m/f ratio 17/34, median age 57 y, range 27-76). The most frequent PM was breast cancer (15 cases), followed by NHL (9) and uterus cancer (7). The median time from PM diagnosis to sAPL was 36 months (range 8-366). PM was treated in 14 cases (27%) with surgery alone. In the other 37 cases (73%), pa-

tients received chemotherapy (10 cases, 20%), radiotherapy (17 cases, 33%) or chemo-radiotherapy combination (10 cases, 20%). M3 variant was found in 7 patients. Thirty-seven patients performed a cytogenetic study: 3 patients had a normal karyotype, 31 had t(15;17); in 3 cases failed. Molecular biology study was done on 36 patients: 21 were BCR1 positive, 3 BCR2 positive and 10 BCR3 positive. In 2 patients it failed. Other 15 patients did not perform molecular biology study. On the whole only in 6 patients diagnosis of M3 was based on morphological criteria only. All patients received a treatment for sAPL: 35 patients were treated with AIDA protocol (idarubicin plus ATRA), 8 with ATRA alone, and 8 patients received chemotherapy including anthracyclines plus cytarabine. Forty-three patients achieved a CR (84%) and 8 patients died in induction (16%). The median duration of CR was 27 months (2-128) and the median overall survival was 27 months (0-130), but the median survival of patients who achieved CR was 29 months (4-130). At the time of analysis 33 patients were alive in CR. Nine patients relapsed and died (2 for hemorrhage, 4 for APL, 2 for infection; one patient developed melanoma). One patient was lost at follow-up. **Conclusion:** Prognosis of acute leukemia following another malignancy is characterized by a bad prognosis. In our serie we observed that, contrarily to the other sAML, the CR rate and the outcome of sAPL is similar to that observed in primary APL.

## CO08

### ACUTE MYELOID LEUKEMIA FOLLOWING ESSENTIAL THROMBOCYTHEMIA (ET)

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Two-hundred eighty-four ET patients were studied at our Institute in a fourteen years period. At the time of diagnosis all cases had a normal chromosome pattern and all were ABL/BCR negative on RT-PCR analyses. All the patients received pipobroman (25 mg/day as starting dose) as first line therapy. Twenty-four cases did not respond to this treatment and therefore 19 received

HU (1g/day) and 5 busulfan (4 mg/day). Ten patients developed AML after a median follow-up of 104 months. Among them 5 had received only pipobroman, 2 pipobroman and HU and 3 pipobroman and busulfan. Seven cases yielded a suitable number of metaphases and all of them showed complex rearrangements involving more than three chromosomes. Conventional cytogenetics detected a 17p deletion in 2 cases. In order to establish the incidence of this structural abnormality, frequently observed in AML following ET we decided to perform FISH analyses with a p53 dyoxigenated probe (red spot) on metaphase and interphase cells. This probe was applied in association with chromosome 17 specific centromeric probe (green spot) in order to evaluate the incidence of false positive/false negative results. A del(17)(p11) was observed in 4/7 cases with a suitable number of metaphases but in 7/10 cases in whom interphase cells only were examined. Morphologically the Pelger-Huet anomaly and vacuolated neutrophils were observed in the peripheral blood of 6 cases. As far as the treatment received is concerned del(17p) occurred in the 4/5 cases who had been administered pipobroman only and in 2 treated with pipobroman and HU. Our data indicate that del(17p) is frequently observed in AML following ET and is not related to previous treatment.

### **C009**

#### **ACUTE LYMPHOBLASTIC LEUKAEMIA(ALL) IN THE ELDERLY: RESULTS OF TREATMENTS IN A COHORT OF 106 PATIENTS**

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In the past yrs ALL in the elderly was considered a rare disease, characterized by a poor prognosis, since age in itself represents one of the most important prognostic factors negatively influencing both the CR rate and disease outcome. In this retrospective study the clinical characteristics, the types of treatment and disease outcome

of 106 older (>60yrs) ALL pts, observed in 3 Centers from January 1969 to March 1999, are discussed. Of 105 pts - 43 males/63 females, median age 68.5 yrs, range 60-90 yrs- median haematological parameters, at diagnosis, were: Hb 9 g/dl, WBC count  $11.8 \times 10^9/l$ , Plts  $80. \times 10^6/l$ . Immuno-phenotype was evaluable in 84pts: 71 were B-lineage ALL, 1S(Ig)+, 5 T-ALL, 6My+ALL and 1 Stem Cell Leukaemia. Cytogenetics was available in 27 pts: t(9;22)(q34; q11), and t(4,11)(q21; q23) were found in 7 and 3 pts respectively; 9 pts were BCR/abl+ (4 p210+, 3 p210/190+, 2p190+), 3 ALL/AF4+. At onset lumbar puncture (LP) was done in 49 pts, CNS involvement was present in 5. As induction pts were subsetted in 2 groups: group A included 58 pts- median age 71yrs - treated with *palliative* 2 drug (VCR+PDN) induction, group B 48 pts- median age 66 yrs- treated with *intensive* induction including Anthracycline. From 1983 these pts were enrolled in the Gimema adult ALL trials: 0183(16), 0288(19), 0394(1), 0496 (2). CNS prophylaxis - LP± cranial Rx, systemic ID methotrexate- was applied in 44 pts. During induction Growth Factors were used in few cases, >50% of pts were treated as out-pts.

	TOT.	CR(%)	REFR.	I.D.	CCR median (mos)	SURV median (mos)
	106	71(67)	15	20	6.2	7.5
PALLIATIVE	58	32(55)	12	14	5.6	8.3
INTENSIVE	48	39(81)	3	6	8.0	9.5

During follow-up 78% and 66% of pts relapsed in group A and B respectively. As of April 1999 16 pts - 4 in group A and 12 in group B- are alive: 13 in 1st CCR from a median time of 20 mos, 1 (p210+) in 2nd CR, 1 (p190+) in 3th CR and 1 with active disease. The median age is increasing in the western countries, an increased incidence of ALL could be occurred in the >60 yrs age population; thus the therapeutic strategy become a prominent issue in the yrs to come.

## **MOLECULAR BIOLOGY AND CYTOGENETICS**

### **CO10 SUPERIORITY OF ALLOGRAFTING OVER AUTOGRAFTING IN TERMS OF MOLECULAR REMISSIONS IN MULTIPLE MYELOMA PATIENTS**

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We have planned a molecular monitoring of minimal residual disease (MRD) in patients achieving complete remission (CR) after autologous or allogeneic transplantation of hematopoietic cells. Clonal markers based upon the rearrangement of IgH genes were generated for each patient and used for PCR detection of MRD. Fifty-one patients entered the program and 36 achieved CR. MRD analysis has been performed on 29 patients (15 autologous and 14 allogeneic) having a molecular marker. Our data show that molecular remissions are rarely achieved (7%) with high-dose (HD) chemotherapy followed by single or double autografting. In addition, we give a further demonstration that virtually all peripheral blood progenitor cell (PBPC) and bone marrow harvests contain residual myeloma cells even when the collection was scheduled after repeated courses of high-dose chemotherapy. All patients reinfusing PCR-positive harvests remained positive, and 8 of 13 had already a relapse. Two patients were autografted with PCR-negative harvests: one is in clinical and molecular remission; one suffered an extramedullary relapse 25 months post-transplant. In the allografting setting, a higher proportion of patients (50%) achieved the molecular remission; there were 3 relapses, two in the PCR-positive and one in the PCR-negative group. The sizeable fraction of patients achieving molecular remission after PBPC allografting is a promising finding in an incurable disease. Further studies on a larger panel of cases

are required to clarify the clinical relevance of molecular remission in myeloma patients.

### **CO11 MOLECULAR ERADICATION OF MULTIPLE MYELOMA IS POSSIBLE AFTER ALLOGENEIC AND AUTOLOGOUS TRANSPLANTATION OF HEMATOPOIETIC CELLS**

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To assess the role of autologous and allogeneic bone marrow transplantation of hematopoietic cells in patients with multiple myeloma (MM), we planned molecular monitoring of minimal residual disease (MRD) for patients in complete clinical remission (CR) after auto- or allo-transplantation. Clonal markers based upon the rearrangement of immunoglobulin heavy-chain genes were generated on 44/52 MM patients who achieved CR (14 allogeneic, 13 single and 17 double autografting). In the allografting setting, 26/68 (38.2%) patients achieved CR: of 14 patients having a molecular marker, 7 patients (10.3%) achieved molecular remission (MCR). In the autografting setting, a total of 36/161 (22.3%) patients achieved CR and were divided in different groups based on autotransplantation procedures. 82 patients were submitted to high-dose chemotherapy followed by single autografting; 71 of them (subgroup A) received a single un-manipulated autograft: 8 patients achieved CR (11.2%) and 6 of them were studied by patient-specific marker: 1 achieved MCR (1.4%). 11 patients (subgroup B) received a single double-selected autograft (CD34+/Blin- cells): 7 achieved CR (1 non-secretory) (63.6%) and all were studied by patient-specific marker: 1 achieved MCR (9.1%). 79 patients were submitted to double autografting: 62 patients (subgroup C) undergone double un-manipulated autografting and 15 of them achieved CR (24.1%); 12 were molecularly studied and 1 patient obtained MCR (1.6%). 17 patients (subgroup D) were re-infused with selected

apheresis (CD34+ cells): 6 of them achieved CR (35.3%) and 5 were molecularly studied: 1 patients obtained MCR (5.9%). We showed that virtually all un-manipulated peripheral blood progenitor cells harvests contain residual myeloma cells. Only few CD34+ and CD34+/Blin- grafts were PCR negative.

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## C012

### REAL-TIME PCR FOR QUANTITATIVE EVALUATION OF RESIDUAL DISEASE IN MULTIPLE MYELOMA (MM) USING THE IMMUNOGLOBULIN HEAVY CHAIN (IgH) GENE REARRANGEMENT

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Despite intensified treatments, patients with MM have shown a nearly constant persistence of PCR-detectable disease. Therefore qualitative PCR analysis has no prognostic significance and quantitative approaches are probably required to identify high-risk patients. Real-time PCR is a novel quantitative method for PCR analysis that proved effective for MRD detection when chromosomal translocations are used as clonal markers. In this study, we developed a real-time PCR approach based on the IgH rearrangement and assessed its effectiveness in 31 MM patients. Because of the high cost of producing patient-specific reporting probes, VH family-specific consensus probes were used in association with tumor-specific primers. A number of mismatches between clonal IgH sequences and consensus probes could not be avoided. However, probe effectiveness was easily predictable by the number and quality of such mismatches. We demonstrate that few probes allow successful real-time PCR in 100% of patients. Our data show that shorter amplicons allow more sensitive and effective real-time PCR. Sensitivity has been assessed for the whole panel of patients and is around  $10^{-4}$ . Finally, assay reproduc-

ibility and accuracy were extensively evaluated, in order to validate our method. Reproducibility was assessed by performing real time PCR on 22 DNA samples in two different rounds of amplification. At each amplification, three replicates were generated. Good agreement was observed (correlation > 0.98) both within the same run and in different runs. Real-time PCR has also shown good accuracy. In the presence of 50 or more target copies, our method could easily discriminate two fold differences in tumor contamination. In addition, dilution experiments with cell lines showed a close correlation between calculated and expected values. Our data thus indicate that real-time PCR using the IgH rearrangement is feasible, accurate, reproducible and not exceedingly expensive. We plan to use this approach to evaluate the kinetics of residual disease in a panel of patients with both MM and non-Hodgkin lymphoma with persistent PCR detectable disease after auto- and allograft transplantation.

## C013

### PATIENT'S SPECIFIC PRIMERS FOR RESIDUAL DISEASE ASSESSEMENT IN CLL AUTOGRAFTING

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Variable regions (VDJ) of the Ig heavy chain genes rearrangement are clone-specific and can be used as tumor marker in B neoplasias. Thirty patients with advanced stage chronic lymphocytic leukemia (CLL) were enrolled in an autograft program with purified CD34 + cells. Minimal residual disease (MRD) was evaluated in peripheral blood stem cells (PBSC) harvest, and in the bone marrow after autografting. The molecular rearrangement of the IgH genes was studied by PCR using primers specific to the framework 3 region (FR3) with a consensus primer from joining region (JH) (22 patients). The amplification with the FR3 primer failed in a single patient and it was necessary the used a panel of primers specific to the framework 1 region (FR1) of the different VH families, together with JH primer. In order to identify the nucleotide sequence of the rearranged variable region

VDJ, and then design tumor-specific primers, the sequence of the third complementary region (CDR3) was obtained by direct sequencing the clonal VDJ-PCR product. MRD was detected using patient specific CDR3 and JH primers. In 23 of 24 patients studied (96 %) a molecular marker was available. All patients had a PCR-positive harvest before and after CD34+ cells immunoselection (13 patients). After autografting 7 out of 11 patients, studied with specific-primers, obtained a molecular remission. Five patients were stable PCR negative (3-24 months), the other 2 became PCR positive at 18 and 36 months respectively, with none clinical evidence of relapse. The sensitivity of the method was around  $10^{-5}$  cells. High-dose chemotherapy is able to provide molecular remission in CLL, but its role in terms of disease free survival remains unknown.

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#### **CO14**

### **CLINICAL VALUE OF RT-PCR MONITORING OF RESIDUAL DISEASE IN PATIENTS WITH t(4;11) ACUTE LYMPHOBLASTIC LEUKEMIA**

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Twenty-three patients (21 adults and 2 infants) with ALL1/AF4-positive ALL patients were prospectively monitored by RT-PCR between January 1992 and February 1999. At presentation, a rearranged configuration of the ALL1 gene and/or a t(4;11) translocation were present in all cases. Following high-dose intensive induction and consolidation chemotherapy without bone marrow transplantation, all patients achieved a complete hematologic remission. By nested RT-PCR (sensitivity  $10^{-4}$ ), conversion to PCR negativity was observed in 10 cases (43%). All 13 patients who remained PCR positive relapsed at a median time of 4 months (range 1 - 20). Of the 10 patients who attained conversion to PCR negativity, 5 converted again to PCR positivity within 1 to 14 months. All 5 progressed to hematologic relapse at 2, 3, 4, 7 and 7 months, respectively, from the reappearance of the ALL1/AF4 transcript. For patients who never con-

verted to RT-PCR negativity, the actuarial probability of relapse and survival was 100% and 0% at 14 and 24 months, respectively. By contrast, for the 10 patients who reached a molecular remission, relapse and survival rates were 71% and 53% at 84 and 100 months, respectively. A statistically significant difference between the two groups was observed with regard to the actuarial survival rate ( $p < 0.005$ ). Altogether, this study represents the first prospective analysis of residual disease monitoring carried out in a substantial group of t(4;11) ALL patients. Our results emphasize the clinical relevance of RT-PCR based methods for the monitoring of minimal residual disease in this leukemic subset.

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#### **CO15**

### **SERUM-FREE TRANSDUCTION OF MOBILIZED BLOOD CD34+ CELLS, AS PART OF A CLINICAL GENE MARKING PROTOCOL**

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In the context of a hematopoietic cell gene marking program in patients undergoing high-dose chemotherapy with autografting of mobilized blood progenitors, we developed an efficient short-time CD34+ cell transduction method, using clinical-grade culture reagents and serum-free culture medium. Selected CD34+ cells were transduced with a retroviral vector encoding for the truncated form of the human low-affinity receptor for nerve growth factor ( $\Delta$ LNGFR), in the presence of clinical-grade retronectin. The time of cell manipulation was 3.5 days, and included a 24-hr cell prestimulation, one 12-hr transduction, and a 48-hr culture period after transduction. Gene transfer rate was assessed by indirect flow cytometric evaluation of surface  $\Delta$ LNGFR expression. During the procedure the cells were cultured in X-VIVO10 medium, without serum and with clinical-grade cytokines (thrombopoietin, Flt3-ligand and steel factor) at 50 ng/ml concentration. We have compared this strategy to a conventional transduction procedure, that used the same conditions except for the utilization

of IMDM supplemented with 10% FCS. Results are reported in the Table:

	Day 0 CD34+ cells (x10e3)	Day 3.5 CD34+ cells (x10e3)	Percent transduction	Day 3.5 transduced cells (x10e3)	Percent transduction of input cells
X-VIVO Serum-free	653	1,135	14.8	167.9	25.7
IMDM 10% FCS	587	722.2	16.1	116.2	19.7

Despite of a comparable rate of transduction, serum-free conditions resulted in higher absolute numbers of transduced CD34+ cells (167 vs 116x10e3), due to an increased CD34+ cell expansion rate, with a favorable balance between rate of transduction of CD34+ cells and the maintenance of an undifferentiated cell phenotype. We conclude that a short-time serum-free transduction procedure is not only feasible for clinical purposes, but also increases overall gene transfer rate in comparison to conventional methods.

## CO16 NK CELL RECOGNITION OF HETEROLOGOUS GENES USED FOR GENE THERAPY

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NK cells express receptors (KIR) which, upon interaction with their MHC class I ligands, produce a signal inhibiting killing of the autologous cell. Thus, NK cells are activated in response to the missing expression of self class I molecules on target cells, such as some allogeneic cells (see abstract by Ruggeri et al. in this meeting). KIR also distinguish peptides. Consequently, NK cell recognition of class I alleles on target cells is prevented, and NK lysis is triggered, by amino acid substitutions along protective peptides loaded onto class I molecules (Malnati M. et al, Science 1995). Replacement of self peptides with endogenously synthesized viral peptides may trigger the NK cell killing of virus-infected cells (Mandelboim O. et al., PNAS 1997). This study investigated the role of NK cells as effectors of an immune response against autologous cells modified by gene therapy. T lymphocytes were transduced with LXS

a retroviral vector adopted for human gene therapy which carries the selectable marker gene neo, and the autologous NK response was evaluated. We found a) infection with LXS makes cells susceptible to autologous NK cell-mediated lysis, b) expression of the neo gene is responsible for conferring susceptibility to lysis, c) lysis of neo expressing cells is clonally distributed and mediated only by NK clones which exhibit HLA-Bw4 allele specificity and bear KIR3DL1, a Bw4-specific NK inhibitory receptor, and d) the targets are cells from HLA-Bw4+ individuals. Finally, neo peptides anchoring to the Bw4 allele HLA-B27 interfered with KIR3DL1-mediated recognition of HLA-B27, i.e., they trigger NK lysis. Moreover, neo gene mutations preventing translation of 2 of the 4 potentially non-protective peptides reduced KIR3DL1+ NK clone-mediated autologous lysis. Thus, individuals expressing Bw4 alleles possess an NK repertoire with the potential to eliminate autologous cells modified by gene therapy. By demonstrating that NK cells can selectively detect the expression of heterologous genes, these observations provide a general model of the NK cell-mediated control of viral infections (J Exp Med 1999, in press).

*Supported by Telethon*

## CO17 CO-EXPRESSION OF MET AND ITS LIGAND HEPATOCYTE GROWTH FACTOR (HGF) IN HHV-8+ PRIMARY EFFUSION LYMPHOMA

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Primary effusion lymphoma (PEL) is a peculiar category of non-Hodgkin lymphoma (NHL) characterized by consistent infection by HHV-8. PEL selectively grows in liquid phase in the serous body cavities with no formation of solid tumor masses. The bio-

logic basis of this growth pattern is unclear. Previous studies suggested that PEL tumor cells reflect a pre-terminal stage of B-cell differentiation (pre-plasmacells). To corroborate this hypothesis, we have studied PEL (n=13) for co-expression of MET and its ligand HGF, which, in the hematopoietic system, are selectively co-expressed by multiple myeloma neoplastic plasmacells. For comparison, a panel of high grade B-cell NHL composed of B-lineage diffuse large cell lymphoma and Burkitt lymphoma (n = 34) was also tested. Expression of MET was analyzed by multiple assays, including reverse transcriptase-polymerase chain reaction (RT-PCR), flow cytometry, immunocytochemistry (ICC), Western blot, and *in vitro* tyrosine kinase activity assay (TK assay). Expression of HGF was analyzed by RT-PCR, scatter assay, and ELISA. All PEL tested co-expressed MET and HGF by RT-PCR. Co-expression of MET and HGF mRNA was selective for PEL and scored negative in other B-cell NHL types. Flow cytometry and/or ICC analysis demonstrated MET protein expression in selected PEL samples and analysis by TK assay demonstrated that the MET receptor expressed by PEL is functionally active. Western blot analysis showed a basal constitutive phosphorylation of MET in all PEL analyzed, suggesting constitutive receptor activation. Scatter and ELISA assays demonstrated that PEL cells release functionally active HGF protein (range: 0.5 - >10 ng/ml). *In vitro* stimulation of PEL cells with HGF induced a rapid increase of phosphorylation of the MET 145 kD b-chain, confirming functional integrity of the MET/HGF loop. The implications of these data are twofold. First, co-expression of MET/HGF is a selective feature of PEL among NHL and corroborates the hypothesis that PEL is histogenetically related to pre-terminally differentiated B-cells. Second, because HGF induces proliferation and motility, the MET/HGF signal pathway may affect the biologic properties and growth pattern of PEL.

## CO18 CONSTITUTIVE EXPRESSION AND TYROSINE PHOSPHORYLATION OF SHC PROTEINS IN CHRONIC MYELOGENOUS LEUKEMIA

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Shc cytoplasmic proteins, including p66<sup>Shc</sup>, p52<sup>Shc</sup>, p46<sup>Shc</sup>, are among the targets through which growth factor receptors transmit mitogenic signals. Shc proteins become phosphorylated upon activation of both receptor tyrosine kinases (RTKs) and surface receptors that have no intrinsic TK activity but can signal by recruiting and activating cytoplasmic TKs. Unlike parental cells, fibroblasts overexpressing a human Shc cDNA acquire partial independence from exogenous growth factors, show anchorage-independent growth and form tumours in nude mice. Using continuous leukemic cell lines, Shc proteins have been shown to be substrates of BCR/ABL fusion proteins. In this study, we investigated the expression and tyrosine-phosphorylation status of Shc proteins in chronic myelogenous leukemia (CML) primary cells. CD34<sup>+</sup> cells from CML in blast crisis (n = 11) as well as CD34<sup>+</sup> and CD34<sup>-</sup> cells from CML in chronic phase (n = 4) were studied. Normal CD34<sup>+</sup> and CD34<sup>-</sup> cells from healthy donors were also analyzed. Shc isoforms, including p52<sup>Shc</sup>, p46<sup>Shc</sup> and p66<sup>Shc</sup>, were demonstrated to be expressed and strongly tyrosine-phosphorylated in CML in blastic phase and in CD34<sup>+</sup> cells of CML in chronic phase. In contrast, Shc proteins were not phosphorylated in normal marrow-derived CD34<sup>+</sup> cells which showed low or barely levels of Shc proteins tyrosine-phosphorylation. The lack of tyrosine-phosphorylation of Shc proteins in normal CD34<sup>+</sup> cells reflected a inactive functional status since both Shc expression and tyrosine-phosphorylation were instead found in the normal peripheral blood CD34<sup>+</sup> cells from G-CSF treated samples. A co-immunoprecipitation of Shc proteins with p210<sup>BCR/ABL</sup> or p190<sup>BCR/ABL</sup> but not with normal p145<sup>ABL</sup> was observed in CD34<sup>+</sup> leukemic cells. Tyrosine-

phosphorylation of the three Shc isoforms was present in the immunoprecipitates. These results suggest that Shc protein phosphorylation is an important step of activation of signalling pathways in CML CD34<sup>+</sup> cells; this behaviour could create a cascade of oncogenes activation beginning with p210<sup>BCR/ABL</sup> and p190<sup>BCR/ABL</sup> constitutive phosphorylation which in turn activate Shc proteins. The nature of adaptor proteins of Shc isoforms may facilitate to engineer novel molecules to inhibit their functions, thus blocking the dysregulated proliferation triggered by BCR/ABL by sparing CD34<sup>+</sup> normal hematopoietic precursors.

## MULTIPLE MYELOMA

### **C019 CHROMOSOMAL ABERRATIONS IN NEWLY DIAGNOSED MULTIPLE MYELOMA**

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Cytogenetic studies in multiple myeloma (MM) are often limited, because of difficulties to obtain a sufficient number of evaluable metaphases, due to the low proliferative activity of neoplastic clone. Up to now, few studies have assessed the impact of specific abnormalities on prognosis and focused on short survival for patients with aberrations of chromosome 13 (monosomy or partial deletion) and 11q13 (partial deletion or translocation). We have studied the karyotypic pattern of 54 newly diagnosed patients with MM, enrolled onto myeloablative therapy "Bologna 96" protocol. An abnormal karyotype was detected in 21 patients (42.9% of evaluable patients). Almost all patients showed a complex pattern, with hyperdiploidy in 5 patients (23.8%), pseudodiploidy in 9 patients (42.9%) and hypodiploidy in 7 patients (33.3%). The number of affected chromosomes ranged from 1 to 17 (median number 5), with at least one trisomy in 47.6%, one monosomy in 42.9% and one translocation in 76.2% of patients with abnormal karyotype. The most common numerical aberrations were chromosomes 3, 5, 9 e 15 trisomies and chromosome 13 monosomy; while the structural aberrations involved mostly 14q, 1p, 11q, 1q, 16p, 12p. The involvement of 14q32 represented the most common abnormality in MM. We have found a cytogenetic involvement of the 14q32 in about 30% of abnormal cases; these included 3 patients with t(11;14) (q13;q32) and 1 patient with a variant t(1;11;14) (q21;q13q32). Preliminary studies of evaluable patients, at least after the first phases of therapeutic protocol, the response rate was 25% in the patients with chromo-

some 13 aberrations and 46.7% in the patients with normal karyotype. No patient with chromosome 13 aberrations is in remission, while 27.3% of the patients with diploidy and 7.1% of the patients with other abnormalities are in remission. Further observations of follow-up of these patients are necessary to define the possible role of chromosomal aberrations on prognosis in MM.

### **C020 LOSS OF TCR DIVERSITY IN MULTIPLE MYELOMA PATIENTS AFTER HIGH- DOSE CHEMOTHERAPY AND PERIPHERAL BLOOD PROGENITOR CELL TRANSPLANTATION**

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The remission phase is currently considered as the most appropriate setting for delivery immunotherapy-based regimens. However, little is known about the T-cell immune competence status of MM patients in first remission after high-dose chemotherapy and peripheral blood progenitor cell (PBPC) transplantation. We have investigated the overall complexity of the TCR repertoire expressed by MM in first remission by estimating the reciprocal usage of functional BV gene segments and measuring in each of them the distribution of the CDR3 region length. Starting from cDNA samples, we used a combination of two PCR reactions, the second one named run-off and made with fluorescent oligonucleotides; then, the results have been submitted to a software analysis. On average, the 33.6% of the total TCRBV repertoire in each individual MM showed an oligoclonal CDR3 length distribution vs the 3.2% in the controls. The TCRBV repertoire analysis was extended to MM patients at diagnosis and patients with monoclonal gammopathy of undetermined significance (MGUS). On average, oligoclonality involved the 18.0% and 5.5% of the whole TCRBV repertoire in MM patients at diagnosis and MGUS, respectively. Thus, evolution from MGUS to overt MM is associated with a loss of TCR

diversity, while the further loss of TCR diversity observed in the remission phase is likely due to the transplantation procedure itself. Thus, any immunotherapy-based approach delivered in the remission phase should be aimed at: 1) recruiting tumor-specific T-cell effector clones; 2) recovering the overall complexity of the TCR repertoire.

## **CO21 CLINICAL VALIDATION OF DIAGNOSTIC CRITERIA FOR LOW RISK IgG MGUS**

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During the course of a recent investigation of 386 patients with MGUS monoclonal gammopathy (Blood: 87, 3, 912, 1996), we defined the hematological variables characterising a group of patients at very low risk of evolution (low-risk MGUS), in whom a non-invasive diagnostic approach and a relatively contained follow-up could be adopted. We here report the follow-up data relating to a group of patients with low-risk IgG MGUS, with the aim of validating our previous proposal in terms of the risk of evolution into multiple myeloma (MM). The diagnostic criteria of low-risk IgG MGUS were as follows: serum MC  $\leq 1.5$  g/dL; absence of Bence-Jones proteinuria; normal serum polyclonal Ig levels, hematic crisis and renal function; absence of symptoms. Since 1996, the patients satisfying these criteria at diagnosis have not undergone skeletal radiology or bone marrow aspiration; the frequency of clinical and hematological examinations is four-monthly in the first year, six-monthly in the second, and annually thereafter. The frequency of evolution into MM was evaluated in 178 patients with low-risk and 140 with non low-risk IgG MGUS. The distribution of the main clinical variables were as follows (low-risk vs non low-risk): M/F ratio: 0.93 vs 0.73; median age: 59 (21-84) vs 61 (20-86) years;  $\kappa/\lambda$  ratio: 1.86 vs 1.78; median serum MC (g/dL): 1.2 (0.2-1.5) vs 1.9 (0.5-3.5). The median follow-up was 80 (24-240) vs 66 (12-156) months. Only one patient

in the first group developed symptomatic MM (8 years after diagnosis) vs. 16 in the second group (4 stage I, 12 stage II-III; 6 deaths) ( $p < 0.001$ ). These data support the diagnostic definition of low-risk IgG MGUS and, in such cases, justify a non-invasive diagnostic approach.

## **CO22 PROSPECTIVE RANDOMIZED STUDY OF MELPHALAN + PREDNISONE (MP) VS MELPHALAN + PREDNISONE + IDARUBICIN (MIP) IN NEWLY DIAGNOSED MULTIPLE MYELOMA PATIENTS**

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Combinations of anticyclines and alkylating agents are widely used in the treatment of multiple myeloma (MM). In comparison to Doxorubicin and Daunorubicin, Idarubicin (IDA) is less cardiotoxic and its intracellular metabolism is relatively independent on multiple drug resistance mechanisms. Furthermore, its oral formulation possesses an acceptable bioavailability, thus rendering the drug suitable for outpatient treatments. Aim of this study was to evaluate the efficacy and toxicity of an IDA-based intensified induction therapy (MIP) compared to classical MP induction, in newly diagnosed MM patients aged  $> 60$ . Between October '95 and April '99, 308 patients entered the study; treatment is still ongoing in 72 patients, while 236 (112 male, 124 female, median age 69 years) are presently evaluable. 155 patients were enrolled in the MIP arm (Melphalan 10mg/sqm/day + Prednisone 80mg/sqm/day + IDA 5mg/sqm/day, day 1  $\rightarrow$  4/month x 6 courses) and 81 in the MP arm (Melphalan 10mg/sqm/day + Prednisone 80mg/sqm/day, day 1  $\rightarrow$  4/month x 6 courses). At present, 178 patients have completed the protocol, while in 58 patients treatment was stopped (25% MIP, 15% MP, 3.8% early death  $< 3$  months). Clinical-hematologic characteristics were similar in the two groups of patients. Median treatment duration was 160 days for MIP and 166 days for MP, with a dose intensity index of 85% and 81% re-

spectively. All the patients that were enrolled to treatment were considered evaluable (intention-to-treat); a response greater than 50% was obtained in 51% of cases (MIP = 49%, MP = 55%,  $p = ns$ ), 16 patients (6.7%) showed a complete remission with disappearance of M component at immunofixation (MIP = 8%, MP = 5%,  $p = ns$ ). Patients enrolled in MIP protocol showed significantly lower PMN counts after treatment (WHO grade 3-4 neutropenia after courses 1, 2, 5 and 6;  $p < 0.05$ ); they have also experienced a higher incidence of febrile episodes (43% vs 23% in MP group,  $p = 0.009$ ). Median survival is 9 months and 12 months for patients treated according to MIP and MP regimen, respectively ( $p = 0.032$ ). Our present data seem to demonstrate that addition of IDA does not increase the efficacy of classical MP regimen.

### **C023 HIGH DOSE CHEMOTHERAPY AND TANDEM AUTOLOGOUS PERIPHERAL BLOOD STEM CELL TRANSPLANTATION AS FRONT LINE TREATMENT IN SYMPTOMATIC MULTIPLE MYELOMA PATIENTS**

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Since September 1993, 60 newly diagnosed consecutive Multiple Myeloma (MM) patients, less than 65 years old, were referred to our institution to be enrolled in a High Dose Sequential (HDS) chemotherapy program based on 3 cycles VAD followed by Cyclophosphamide (CTX, 7 gr/sqm) + G-CSF (5 mcg/kg/day) for peripheral blood stem cell (PBSC) collection. Patients were then planned to receive autologous PBPC to support tandem sequential transplants (TRX) conditioned with Melphalan (200 mg/sqm) and Melphalan (140 mg/sqm) plus Total Body Irradiation (TBI, 1200 cGy), respectively. Fifty nine patients completed VAD induction therapy while first and second TRX were performed in 57 and 41 patients, respectively. Complete Remission (CR) was obtained in 5 patients (8%) after VAD, in 25 (42%) after the first TRX and 7 additional patients obtained CR after the

second myeloablative procedure (50%). The main results of the study are summarized in the Table

Therapy	N.Patients	Cumulative- =75%	Response =90%	(%) RC
VAD	59	43	28	8
HD CTX	58	52	40	27
1° TRX	57	73	65	42
2° TRX	41	85	73	50

The median time to neutrophil engraftment (more than  $0.5 \times 10^9/L$  neutrophils) after the first and second TRX was observed after 10 and 9 days respectively while a stable platelet recovery (more than  $20 \times 10^9/L$  platelets) was documented at day +10 and +11. As a maintenance treatment, Interferon- $\alpha$  was administered to 24 patients until disease progression or relapse. By an intention to treat analysis and with a median follow-up of 1.9 years (range 0.2-5.4) the actuarial 5 year Event Free Survival (EFS) and Overall Survival (OS) are 37% and 51%, respectively. As comparison, the actuarial 5 year OS of our 57 historical controls treated by conventional chemotherapy programs (MP, VMCP and/or VBAP) is 27%. These results suggest that HDS chemotherapy and tandem autologous PBPC transplantation is a feasible procedure and apparently is associated with a better survival ( $p = 0.03$ ) when compared to conventional treatments.

### **C024 DOSE-INTENSIVE/DOSE-DENSE MELPHALAN WITH STEM CELL SUPPORT (MEL100) IS SUPERIOR TO STANDARD TREATMENT IN MYELOMA PATIENTS**

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Dose intensive chemotherapy with stem cell support has been reported to improve response rate and survival in multiple myeloma patients. Several studies have evaluated the toxicity and efficacy of one or two cycles of melphalan 200 mg/m<sup>2</sup>. In this study we investigate a new dose-intense / dose-dense regimen where repeated infu-

sions of melphalan 100 mg/m<sup>2</sup> (MEL100) were delivered. Clinical outcome of myeloma patients receiving MEL100 was then compared with that of patients receiving melphalan 200 mg/m<sup>2</sup> followed by autologous transplantation (AT) and oral melphalan and prednisone (MP) conventional chemotherapy. After cyclophosphamide (4gr/m<sup>2</sup>) and G-CSF (10 µg/Kg/die) three leukapheresis were performed and harvests cryopreserved. Melphalan 100 mg/m<sup>2</sup> (MEL100) was infused with stem cell support and repeated after 2 months. The third MEL100 course was only delivered to patients who did not reached complete remission after 2 MEL100 courses. Seventy-one myeloma patients up to the age of 74 entered the protocol at diagnosis. After the first course, the median duration of severe neutropenia and thrombocytopenia was 5 and 2 days respectively. Transfusion requirement was needed in 60% of patients. Mucositis was observed in 14% of patients. Fever of unknown origin was the major extra-hematologic toxicity affecting 27% of patients, and requiring hospitalisation in 25%. No cumulative toxicity was observed after the second and third course. The clinical outcome of patients receiving MEL100 was compared with 71 pair matches (median age 64) selected from patients treated at diagnosis with MP to match for age and B2-microglobulin. Patients receiving MEL100 were also compared with historical control of 54 patients treated at diagnosis with single or double AT. Complete remission was 47% after MEL100, 5% after MP, and 50% after AT. Median event-free survival was 34 months in the MEL100 group, 17.7 months in the MP group, and 38.9 months in the AT. Both MEL100 and AT had a significantly longer event-free survival ( $p < 0.0002$ ) than the MP group. Median overall survival was 56+ months for MEL100, 48 months for MP, and 77.4 months for AT. In conclusion, we have demonstrated that MEL100 is a safe and effective procedure. Compared with MP, both MEL100 and AT improved clinical outcome of myeloma patients.

## CO25

### **MULTIPLE MYELOMA: THE NUMBER OF REINFUSED PLASMA CELLS IN PATIENTS TREATED WITH INTENSIFIED CHEMOTHERAPY IS NOT RELATED TO PATIENT PROGNOSIS**

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In Multiple Myeloma (MM), a major concern in autotransplant with PBPC is represented by the presence of contaminating tumor cells always detected in PB and staminoapheresis products by molecular biology techniques. Reinfusion of plasma cells (PC) during autotransplant could be related to disease recurrence in MM. The relationship between the number of reinfused PC, response to chemotherapy and event-free survival (EFS) have been evaluated in 77 MM patients at diagnosis treated with intensified chemotherapy between March 1995 and December 1998. PBPC mobilisation was obtained with cyclophosphamide and G-CSF; patients were then treated with melphalan 100mg/m<sup>2</sup> (MEL100) followed by PBPC support. Two to three courses were administered with a two-months interval. PC were detected and quantitated by cytofluorimetric analysis by labelling cells with monoclonal antibodies. Anti-CD38, CD138, and anti-cytoplasmic Ig were used to identify PC. Median number of reinfused PC was 4.02 x10<sup>6</sup>/Kg (range: 0.5-30.94). No correlation has been demonstrated between the number of reinfused PC and response to chemotherapy: patients reaching CR received 3.75x10<sup>6</sup>/Kg PC, while those with a PR or no response received 5.9 and 3.2x10<sup>6</sup>/Kg PC, respectively. Similarly, no correlation was observed between the number of reinfused PC and EFS. EFS was 40.9 months for patients receiving less than 4.02 x10<sup>6</sup>/Kg PC, 36.4 months for those receiving more than median value ( $p=0.55$ ). Phenotypical analysis of BM and circulating PC present in staminoapheresis collections showed a marked difference in surface antigen pattern: BMPC are predominantly CD45-, CD19-, CD56+, while contaminating PC are CD45+, CD19+, CD56-; proliferative activity of PC, analyzed by cytofluorimetric techniques, was signifi-

cantly higher in contaminating PC than BMPC (18.6% +/-2.7% vs 2.5% +/-1.2;  $p < 0.05$ ). Reinfusion of contaminating plasma cells are not related to patient outcome. Thus, these data suggest that in vitro purging techniques cannot avoid disease recurrence.

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**C026**  
**ALLOGENEIC TRANSPLANTATION IN MULTIPLE MYELOMA: FAVORABLE RESULTS WITH THE USE OF PERIPHERAL BLOOD STEM CELLS**

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In multiple myeloma (MM) allogeneic transplantation is able to induce complete and durable responses, but is accompanied by a high mortality. With the aim of reducing TRM, we have employed allogeneic peripheral blood stem cells (PBSC) in 18 patients with MM (M/F=15/3; age 48 a., range 35-55). The donors were HLA-identical siblings. For PBSC mobilization they received G-CSF or GM-CSF followed by G-CSF. The characteristics of the patients were the following: IgG=12, IgA=1, IgD=1, BJ=3, non-sec=1; stage II=1, stage III=16, PC leukemia =1. Interval from diagnosis = 8 months (range 4-39). 17 patients are evaluable for transplant results. At the time of allograft 3 were in CR, 6 in PR, 6 were refractory and 2 in progression. Conditioning was busulfan-melphalan (N=16) or busulfano-cyclophosphamide (N=1). The graft contained  $13.1$  (range 4.4-24.1)  $\times 10^6$ /kg CD34+ and  $2.3$  (range 0.9-7)  $\times 10^8$ /kg CD3+ cells. GVHD prophylaxis was CSA-MTX in all. Engraftment was rapid, with  $0.5 \times 10^9$ /L PMN and  $50 \times 10^9$ /L platelets on day 12 and 13, respectively. 10 patients developed acute GVHD  $\geq$  grade II (grade II in 7, grade III in 2, grade IV in 1). Following transplantation, 13 patients (86%) attained a CR, one of them 13 months following transplantation. 5 patients have died, at 1-5 months, and 10 are in CR

at 6-42 months (median 25) from the allograft. In 8 of 10 patients a negative PCR assay for IgH gene rearrangement (patient-specific probes) was demonstrated in at least one post-graft bone marrow examination. Overall survival is 70%, and progression-free survival 45% at 46 months. PBSC can make the allogeneic transplantation in MM more easily applicable, but a more extended study is necessary to assess the impact of the method on disease eradication.

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**C027**  
**ALLOTRANSPLANT FOR MM DURING 1988-1998: REDUCED TRANSPLANT-RELATED MORTALITY AND ATTAINMENT OF PROLONGED CLINICAL AND MOLECULAR REMISSION**

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At the Institute of Hematology and Medical Oncology "Seràgnoli", the University of Bologna, 57 pts (median age, 43 yrs; stage III, 63%; refractory or progressive, 57%) have received allogeneic stem cell transplants (allo TX) from HLA-identical sibling donors between 1989 and 1998. Conditioning treatments, the source of hemopoietic stem cells and methods to prevent GVHD were different over the following periods of the study. Years 1989-1993: 21 pts, BU-Cy 4, bone marrow (BM), CsA + MTX. Years 1993-1995: 16 pts; Cy + HDM + TBI, BM, T-cell depl. Years 1995-1998: 17 pts; Cy + HDM + TBI, peripheral blood (PBSC) in 88% of pts, CsA + MTX. Nineteen pts (36.5% of all pts; 47.5% of those evaluable) attained stringently defined CR. Transplant-related mortality (TRM) was 29.6%, with infection being the leading cause of death. The corresponding figures in groups 1 to 3 were 38%, 37.5% and 18%, respectively. Survival was 50% at 2 yrs, 38% at 3 yrs and 33% at 6 yrs. The relapse rate of pts who attained post-TX CR was approximately 50% at 5 yrs. The following variables were significantly related with TX outcome: attainment of CR and overall survival=both

chemosensitive disease and female sex; progression-free survival=chemosensitive disease. Case-matching analysis between a subgroup of 12 pts receiving PBSC-allo TX and a control subgroup of pts who were transplanted with BM revealed a lower TRM and a higher frequency of chronic GVHD in the PBSC subgroup as compared to the BM subgroup (13% vs. 42% and 20% vs. 45%, respectively). Finally, retrospective detection of MRD by ASO-PCR showed persistently negative PCR results in 8 out of 12 pts who attained clinical remission and could be evaluated. Four of these pts remained in continuous clinical and molecular CR for 3 to 9 yrs. It is concluded that allo TX can be actually offered to MM pts at a risk of death comparable to that expected with any other hematologic malignancy. This procedure is associated with prolonged clinical and molecular remission in a certain fraction of pts, especially if they are females and/or have chemosensitive disease at TX.

## HEMOSTASIS AND THROMBOSIS

### **C028** **ACQUIRED VON WILLEBRAND** **SYNDROME (AvWS) IS HIGHLY** **ASSOCIATED WITH LYMPHO-MYELO-** **PROLIFERATIVE DISORDERS:** **REPORT ON 211 CASES OF THE** **INTERNATIONAL REGISTRY OF AvWS**

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ON vWF)

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**Introduction:** Acquired von Willebrand syndrome (AvWS) is a rare acquired bleeding disorder similar to the congenital vWD in terms of laboratory findings. Diagnosis of AvWS can be very difficult and treatment has usually been empirical. **Aims of the study:** a retrospective analysis of the patients with AvWS reported to propose better strategies to identify and characterize new cases of AvWS. **Methods:** A questionnaire, devised to collect specific information on AvWS, was sent to all the members of ISTH. **Results:** Information about 221 patients from 50 Centers world-wide were collected and analyzed by the coordinators. AvWS was associated with lympho-proliferative (LPD,47%) or myelo-proliferative (MPD,19%) disorders, cardiovascular diseases (CVD,13 %), neoplasia (NEO,7 %) and others diseases (OTH,14 %). The results (as % or mean values) are:

AvWS (total,221):	LPD(98)	MPD(40)	CVD(27)	NEO(14)	OTH(32)
sex (% of male)	59	38	56	50	46
age at onset (yrs, mean)	63	46	57	61	62
bleeders (%)	70	38	11	50	50
in follow-up (%)	58	18	59	50	67
vWF:Ag (U/dL, mean)	25	68	120	32	31
vWF:RCo(U/dL,mean)	8	22	68	17	7
fVIII:C (U/dL,mean)	21	33	131	23	25
pos anti-FVIII/vWF (%)	14	2	n.t.	14	12
<b>Effective therapy with:</b>					
ddavp (%)	31	15	7	21	19
fVIII/vWF conc (%)	38	5	7	43	22
immunoglobulin (%)	16	0	0	14	3

**Conclusions:** our data suggest that AvWS

is highly associated with LPD and MPD: therefore a form of AvWS must be suspected when an excessive bleeding occurs in patients with LPD and MPD.

### **C029** **MOLECULAR MECHANISMS** **PREDISPOSING TO ISCHEMIC** **JUVENILE STROKE**

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The main causes of ischemic stroke are atherothromboembolism and thrombotic occlusion of lipohyalinotic small-diameter end arteries. Marked differences are also seen between younger and older patients as to the risk factors of stroke. In juvenile (<40 years) stroke patients, unusual causes like migrainous stroke and mitral valve prolapse appear to be more frequent, whereas in older (>40 years) patients the classical cardiovascular risk factors of hypertension, diabetes, high cholesterol, myocardial infarction, and cardiac arrhythmias are seen. Although 4% of cerebral infarcts in the young can be attributed to hematologic disturbances that predispose to thrombosis, the frequency of cerebral infarcts caused by prothrombotic states is not known. To evaluate the role of gene polymorphisms in the genesis of cerebral infarcts in young patients, we quantitated these mutations in a group of patients with idiopathic cerebral infarction and compared the results with those of healthy control subjects matched for age and sex. We genotyped 164 consecutive patients under 40 years of age with cerebral infarction of undetermined cause. Mutations in FV and FII genes, and polymorphisms in MTHFR, PAI-1 and ACE genes were carried out. Factor V Q506 allele was found in 7 (4.3%) patients compared to that observed in control group (2; 1.2%.  $\chi^2=.001$ ;  $p=ns$ ). Factor II A20210

allele was depicted in 17 (10.3%) patients (in 1 at homozygous form) compared to 3 (1.8%) in controls ( $\chi^2=9$ ;  $p=ns$ ). The C677T transition in the MTHFR gene was found in 72 (44%) at heterozygous state and in 39 (24%) at homozygous state. Genotype frequencies in control subjects were 58 (35%) for heterozygotes and 12 (7.3%) for homozygotes ( $\chi^2=5.19$ ;  $p=0.02$ ). PAI-1 4G allele was detected in 120 (55.2%) patients compared to that found in control group (77, 30.4%;  $\chi^2=5.3$ ;  $p=0.02$ ). The frequency of D allele in ACE gene polymorphism was 61% in patients while in controls ranged 37% ( $\chi^2=4.0$ ;  $p=0.04$ ). Our data show that polymorphisms of the genes of the natural anticoagulant system and vascular vessel wall are more frequent in young individuals suffering from ischemic stroke compared to healthy subjects. From a clinical point of view, these informations would be beneficial for studies which will investigate inherited basis of arterial brain thrombophilia.

### **C030 MODULATION OF THE HYPERCOAGULABLE STATE BY ALL-TRANS-RETINOIC ACID IN PATIENTS WITH BREAST CANCER**

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Activation of the hemostatic system and thrombotic complications are frequent in malignant disease. The differentiating therapy with all-trans-retinoic acid (ATRA) efficiently controls the severe coagulopathy associated with acute leukemias. To evaluate whether ATRA is able to modulate blood clotting activation and thrombotic events also in solid tumors, we have prospectively studied fifty-five consecutive patients with locally advanced operable breast cancer enrolled in a phase Ib Italian study of biological activity and safety of ATRA  $\pm$  Tamoxifen (Tam)  $\pm$  Interferon alpha 2 (IFN). Pre-operatively, patients ( $n = 5$  / group) were treated for 21 days with escalating doses ATRA (15, 45, 75 mg/m<sup>2</sup>/d on alternate days; groups A15, A45, A75)

or ATRA + 20mg/d T (groups A+T) or ATRA + Tam + 3 M IU/d INF (groups A+T+I). Two groups received Tam (group T) or INF (group I) alone, respectively. Plasma samples from all subjects were obtained at baseline, on days 7, 14 and 21 of therapy and 30 days after operation (performed within 28 days from start). Parameters measured were: 1. markers of hypercoagulation (TAT complex, F1+2, D-Dimer, FVIIa); 2. fibrinolysis proteins (t-PA, PAI-1, and euglobulin lysis area [ELA]); and 3. endothelium activation markers (thrombomodulin [TM] and von Willebrand factor [vWF]). At baseline the overall cancer patient group showed levels of TAT, F1+2, D-Dimer, FVIIa and PAI-1 significantly greater than those of a control non-cancer subject group. During treatment, the hypercoagulation markers were not different between A15, A45, A75 groups, but after operation were decreased in A45 and A75 groups compared to A15. In A+T groups, there was a significant decrease in TAT, D-Dimer and FVIIa levels compared to the group on T alone (F1+2 and FVIIa;  $p<0.05$ ), with no changes in t-PA, PAI-1, TM and vWF levels. IFN addition (groups A+T+I) did not affect the plasma markers of hypercoagulation within and between groups, but significantly increased the endothelial damage markers (eg., A+T+I group: vWF and TM on day 0 vs day 21,  $p<0.01$ ). In conclusion the results of this study suggest that ATRA given pre-operatively to breast cancer patients modulates: 1. the cancer-associated hypercoagulable state, particularly in subjects receiving Tam, and 2. the endothelium activation/damage parameters in patients receiving IFN. They also suggest a reduction in clotting activation post-operatively in subjects receiving the highest doses of ATRA. However no clinically evident thrombotic events were recorded in any of the study groups of patients.

### C031 INCREASED PREVALENCE OF THROMBOSIS IN PATIENTS WITH MULTIPLE CAUSES OF THROMBOPHILIA

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From 1984 to 1999 we studied 275 patients with hereditary thrombophilia belonging to 105 families. 253 (92%) patients showed a single cause of thrombophilia (FVLeiden =149; FII20210=57; PC =13; ATIII =14; PS =20), whereas 22 had two or more associated causes of congenital or acquired thrombophilia (FVLeiden+FII20210=9; FVLeiden+HOCys=3; FVLeiden+LAC=1; FII20210+PS =1; FII20210+PC= 1; PC+HOCys =1; FII20210+LAC=4; FVLeiden+FII20210+HOCys = 1; FVLeiden + LAC+HOCys = 1). In this study we compared the two groups of patients with single or multiple thrombophilic defect for the prevalence, type (arterial or venous) and recurrence of thrombosis and age of onset of the first thrombotic event. The prevalence of thrombosis was significantly higher ( $p<0.0001$ ) in patients with multiple than those with single defect (86.3 % vs 38.3%; vd tabella). The difference remained significant also when the prevalence was calculated only in the family members excluding propositi (75% vs 12%,  $p<0.0001$ ). On the contrary, significant differences between the two groups were not observed for the type of thrombosis (78.9 % venous events in patients with multiple defects, 80.4 % venous events in those with single defect), recurrence (multiple =36.8%; single = 34.02%) and median age of onset of the first thrombotic event (multiple: median = 38 years, range= 23-56; single: median = 31 years, range = 1-70). This study shows an increased thrombotic risk in patients with multiple causes of thrombophilia and, thus, confirms the importance to assess other congenital and acquired thrombophilic factors also in patients with an already diagnosed hereditary thrombophilia.

	Trombosi venose (n° eventi)		Trombosi arteriose (n° eventi)			Totale n.° eventi (prevalenza)
	TVP+EP +TF	cerebrali	addominali	cerebrali	IMA	
s *	70	5	3	12	2	97 (38.3)
m	12	0	3	4	0	19 (86.3)

s = difetto singolo; m = difetti multipli  
TVP = trombosi venosa profonda; EP = embolia polmonare;  
TF = tromboflebite superficiale; IMA = infarto miocardico

### C032 AUTOLOGOUS TRANSFUSION OF CRYOPRESERVED PLATELETS FOLLOWING HIGH DOSE CHEMOTHERAPY AND STEM CELL TRANSPLANTATION

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Thrombocytopenia following high-dose-chemotherapy and stem cell transplantation is usually short and the possibility of supporting patients with cryopreserved au-

tologous platelet concentrates may be considered. To evaluate this technique, we applied it to women undergoing myeloablative therapy for high-risk breast cancer. Platelets were collected by plateletapheresis at platelet rebound following cyclophosphamide administration, frozen and reinfused when platelet count dropped below  $20 \times 10^9/l$  after thiotepa and L-PAM + autologous stem cell transplantation. In a first group of 32 patients, platelets were cryopreserved in 5% DMSO at  $-180^\circ\text{C}$  after computer-controlled rate (CR) freezing. Platelet loss during freezing-thaw-wash procedure was 37%. 28 patients required platelet support: 24 received only autologous platelets, while 4 required additional allogeneic support. Mean corrected count increment (CCI) at 1 hour was  $8.7 \times 10^9/l$  (CCI in 15 control patients supported with fresh allogeneic platelets: 13.3). In vitro release reaction and aggregation of cryopreserved platelets was 60% of fresh allogeneic platelets. In a second group of 14 women, autologous platelets were cryopreserved in 2% DMSO-ThromboSol either by direct insertion in a  $-80^\circ\text{C}$  freezer or by CR freezing and storage in liquid nitrogen. Platelet loss was 50% in both groups. 6 of 7 patients receiving platelets cryopreserved at  $-80^\circ\text{C}$  (mean CCI: 2.0) required additional allogeneic platelet support, while only 1 of 7 patients receiving transfusion of CR frozen platelets required allogeneic transfusion (mean CCI 9.1). With both freezing techniques, platelet aggregation was reduced to 25% of control and 25% of platelets had increased surface expression of activation antigens and reduced expression of GPIb. Membrane GPIIb-IIIa was reduced only in platelets cryopreserved at  $-80^\circ\text{C}$ . In conclusion, autologous transfusion of cryopreserved platelets is feasible and storage in liquid nitrogen with 5% DMSO following CR freezing is actually the method of choice.

### C033 PLATELET TRANSFUSION AFTER BEAM HIGH-DOSE CHEMOTHERAPY IN PATIENTS WITH LYMPHOMA: PROGNOSTIC FACTORS AND GENERATION OF A RISK MODEL

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**Purpose:** to identify risk factors for chemotherapy-induced thrombocytopenia requiring platelet transfusions (PT) in patients (pts) with non-Hodgkin lymphoma (NHL) or Hodgkin's Disease (HD) undergoing autologous stem cell autotransplant (ASCT) after BEAM high-dose chemotherapy (HDC). **Patients and methods:** since May 1989 to March 1999 171 pts (median age 45 years, range 16-67) with NHL or HD were treated with BEAM HDC followed by ASCT. The graft consisted of peripheral blood stem cells (PBSC, 149 pts), bone marrow (BM, 18 pts) or both (4 pts). In 94 pts the transplant was given as part of upfront therapy while in 77 pts was carried out as part of salvage treatment for resistant or relapsed NHL or HD. PT were given prophylactically if platelet count was less than 20.000/mcl and therapeutically whenever necessary. The following prognostic factors were examined: characteristics at presentation [age, gender, histology, stage, performance status, LDH, extranodal sites, previous chemotherapy regimens and cycles, PT before transplant], at transplant time [number of CD34+ cells reinfused, type of reinfusion (BM- vs PBSC vs BM+PBSC), platelet count, state at transplant (CR vs PR vs NR/PROGR)] and after transplant [infections, hemorrhagic events, G-CSF, red blood cell transfusions]. **Results:** 32% of pts in first line and 38% of resistant or relapsed pts were transfused more than 2 times and significant hemorrhagic events (EORTC grade 2) were observed in 23% and 24% of the two groups. The use of G-CSF, infections, platelet count  $<100.000/mmc$ , transplant with BM stem cells, number of CD34+ cells  $< 4 \times 10^6/kg$  and age  $>45$  years were associated with increased need of PT by univariate analysis; only N° of CD34+ cells (0.046), G-CSF (0.009), platelet count less

than 100.000 /mmc (0.000) and source of stem cells (0.006) retained prognostic significance by multivariate analysis. Using these 4 parameters, a risk model was created giving each variable an arbitrary risk coefficient of 1. Thus, the calculated probability of being transfused more than 2 times was 5%, 32% and 89% for pts with 0,1 and more than 1 risk factor respectively. **Conclusions:** The risk index detected in the setting of transplanted lymphoma pts could be useful to identify pts at high risk for chemotherapy-induced thrombocytopenia requiring PT. This group might be suitable for exploring the role of thrombopoietic factors to reduce the need for platelet support.

### C034

#### PLATELET ACTIVATION IN CONCENTRATES FOR TRANSFUSION USE; A STUDY WITH THE EMPLOYMENT OF THE ANNEXIN V

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Previous studies demonstrated that the employment of a recombinant placental protein, the annexin V, might represent a very highly sensitive and specific method to recognize activated platelets. This protein interacts with the prothrombinase-binding anionic phospholipids exposed on platelet surface upon activation. This probe is more sensitive than others previously utilized for in vivo and in vitro detection of activated platelets including monoclonal antibodies like CD63, CD62p and PAC1. In the present study we wanted to apply annexin V test in order to evaluate the platelets activation state in concentrates collected by apheresis, conducted with an Autopheresis Baxter, and in concentrates collected by centrifugation from single donor units. This test has been performed on day 1, in order to establish some differences in preparation techniques, and on day 3 and 5 to evaluate the influence of conservation; furthermore we have evaluated pH, the white blood cell and the platelet count to investigate possible correlations. The percentage of activated platelets has been evaluated by flow cytometry utilizing

a double staining Annexin V FITC/CD41 PE. We performed 119 determinations on 90 platelet concentrates and on 29 apheresis units. The mean value of annexin V on day 1 was  $7.3 \pm 8.3$  in the platelet concentrates and  $4.8 \pm 5.2$  in apheresis units ( $p=n.s.$ ). In the first group the percentage of units with activated platelets  $>10\%$  was 29% while in the second group it was 45% ( $p=0.1$ ). During conservation we have seen a gradually constant increment of the positivity of annexin V and the increment of the value were stronger in the units with high value on day 1. The comparison between the value obtained on day 1, 3 and 5 was performed with variance analysis and showed significant differences ( $p=0.01$ ). At the same time we noted during conservation a progressive reduction in the value of pH (mean value of day 1: 7.4, of day 5: 7.0;  $p=0.008$ ). The application of the linear regression test showed a significant correlation between reduction of pH and increment of annexin V even if a higher value of annexin didn't always correspond to a lower value of pH. Furthermore we have not found a significant correlation between percentage of platelet activation and white blood cell count or platelet count. In conclusion the employment of the annexin V in order to evaluate platelet activation is an easy, simple and sensible method for the periodic evaluation of quality production and conservation of platelet concentrates.

### C035

#### RETICULATED PLATELETS AND GLYCOCALICIN: A NOVEL APPROACH IN THE STUDY OF THROMBOCYTOPENIA

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Thrombocytopenia is a pathological condition consequent to a large variety of causes. It may depend on a decreased platelet production or an increased platelet consumption. The identification of the underlying pathological mechanism is important for a correct clinical, prognostic and therapeutic strategy. Determination of

platelet life-span, evaluation of bone marrow megakaryocytes and platelet morphology are largely used in distinguishing thrombocytopenia due to defective production or accelerated peripheral destruction. The diagnostic value of platelet antibodies is still not completely understood. Recently two new non invasive assays have been introduced: the Glycocalin (proteolytic fragment of GPIb) which seems to be able to evaluate the platelet turn-over and Reticulated Platelet Count (the percentage of young platelet), indicating a shift towards increased thrombopoiesis, therefore a peripheral measure of bone marrow activity. A total of 72 patients was selected for this study (17 Acute ITP, 35 Chronic ITP, 20 Aplastic). We have also studied 60 healthy subject matched for age and sex, as control. The results are summarized in the following table:

	NORMAL (60)	Acute ITP (17)	Chronic-ITP (35)	APLASIA (20)
PLT x10 <sup>3</sup> /ml	235±45	32.8±32.3	75.9±22.9	77±38
PRET %	0.95±0.3	5.05±2.85*	2.56±1.44*	1.0±0.3
GLC mg/ml	0.8±0.2	0.92±0.34	0.92±0.35	0.43±0.31
GLC Index	0.9±0.2	19.7±23.6*	3.1±1.68*	1.28±0.36
Direct Ac anti-plts	-	4 positive	9 positive	-
Indirect Ac anti-plts	-	8 positive	6 positive	-

\* p<0.05

Our results confirm that GC index and PRET% are both helpful in differentiating thrombocytopenia due to increased platelet destruction from the one due to impaired production. Both tests are easy to perform, have a better diagnostic and predictive value than the search of anti-platelet antibodies and may be used in management and follow-up of thrombocytopenia.

### **C036 EVALUATION OF PLATELET ASSOCIATED AUTOANTIBODIES: A PROPOSAL FOR A NEW CLASSIFICATION OF "IDIOPATHIC THROMBOCYTOPENIC PURPURA"**

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Idiopathic thrombocytopenic purpura (ITP) is due to the autoimmune destruc-

tion of platelets by the reticuloendothelial system. However, unlike autoimmune haemolytic anaemia, the assay and the characterisation of autoantibodies have not, until now, clinical relevance in the diagnosis of ITP. Nevertheless, based only on clinical ground, there is the possibility that several forms of thrombocytopenia are classified as "idiopathic" nonetheless they are not secondary to an immune mechanism. In fact even if recent reports have indicated that the platelet antigen immobilization assays are specific for the diagnosis of ITP, in more than 40% of ITP patients a platelet autoantibody was not detectable. We performed a prospective study on 35 patients (F28, M7, 43±19 yrs, 61±37x10<sup>9</sup>/l platelets) in whose an accurate diagnosis of ITP was made by the classical criteria of exclusion. In such patients we measured platelet associated IgG (PAIgG) by flow-citometry and specific associated autoantibodies by monoclonal antigen capture ELISA (MACE) for glycoprotein IIb/IIIa, Ib, Ia/IIa and IV. 20/35 (57%) patients have increased PAIgG, 18/35 (51%) have autoantibodies against platelet specific glycoproteins (5 IIb/IIIa, 4 Ib, 3 IIb/IIIa +Ib, 3 IIb/IIIa + Ib + Ia/IIa, 2 IIb/IIIa + Ia/IIa 1 IIb/IIIa +Ib + Ia/IIa + IV). The relationship between specific platelet autoantibodies and PAIgG positivity was highly significant (p<0.0001). We showed that PAIgG are still useful in ITP and that half patients have no detectable autoantibodies in spite of the use of specific antigen immobilization assay. As in autoimmune haemolytic anaemia, we suggest that the disease of patients with specific platelet autoantibodies should be classified as "autoimmune thrombocytopenic purpura" and that of patients without detectable platelet autoantibodies as "idiopathic".

## **CHRONIC LYMPHOCYTIC LEUKEMIA AND LYMPHOMAS**

### **C037**

#### **ACQUIRED DELETION OF THE ATAXIA TELEANGIECTASIA (ATM) LOCUS IN NON-HODGKIN'S LYMPHOMA: CORRELATION WITH CLINICOBIOLOGICAL FEATURES**

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Cytogenetic and fluorescence in situ hybridization (FISH) analyses were performed, to study the clinicobiologic significance of acquired deletions involving the ataxia-teleangiectasia locus (*ATM*-) in 135 non-Hodgkin's lymphomas (NHL). An hemizygous *ATM*- was seen in 44-88% of cells in 18 cases (13.3%): 5 patients had a B-cell high grade NHL, 8 patients had B-cell low-grade NHL, 5 patients had a T-cell lymphoma. Twelve out of 18 *ATM*- patients had a complex karyotype, 13 out of 18 had more than 90% abnormal metaphases (AA karyotype status); +12, 13q14 deletion or 17p13 deletion were seen in 8, 4 and 4 cases, respectively. Patients with *ATM*- had more frequently a T-cell phenotype ( $p=0.009$ ), complex karyotype ( $p=0.001$ ) and AA karyotype ( $p=0.008$ ) as compared with patients without *ATM*-. Dual color cohybridization of a *BCL1* probe or *TCL1* probe and of the *ATM* probe showed *ATM*- to be possibly a secondary event in 4 *BCL1* rearranged cases and to be an early event in 2 *TCL1* rearranged lymphomas. A highly significant correlation was found between *ATM*- and shorter survival ( $p=0.0002$ ). This cytogenetic lesion maintained its prognostic importance in multivariate analysis ( $p=0.0036$ ), along with complex karyotype ( $p=0.017$ ), performance status ( $p=0.0005$ ), serum LDH level ( $p=0.0055$ ), male sex ( $p=0.001$ ) and splenomegaly ( $p=0.0268$ ). Though possibly not represent-

ing a primary genetic lesion in the majority of cases, acquired *ATM*- has a clinicobiologic importance in NHL, possibly representing a major cytogenetic determinant of outcome.

### **C038**

#### **PERIPHERAL BLOOD CD 38 CELL EXPRESSION PREDICTS SURVIVAL IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA (CLL)**

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The aim of this study was to evaluate the prognostic impact on survival of CD38 expression of peripheral blood lymphocytes in 161 previously untreated CLL patients. All cases fulfilled the recommended diagnostic criteria and showed dim SIg, CD5<sup>+</sup>, CD19<sup>+</sup>, CD23<sup>+</sup> immunological pattern. All patients were prognostically stratified according to Rai and Binet stages and Total Tumor Mass (TTM) score. Rai stages were grouped in 0, I-II and II-IV stages, according to NCI CLL guidelines. Bone marrow biopsy was performed in 113 cases and evaluated in agreement with Rozman criteria. Doubling time (DT) calculated in 115 patients was  $\leq 12$  months in 12 cases only, possibly because more than half patients received first line chemotherapy at diagnosis because of advanced disease; thus, this parameter could not be included in the prognostic evaluation. Lymphocytes of patients younger than 60 years showed significantly lower CD38 percentage mean value as compared to older patients ( $p=0.016$ ). On the other hand, a brighter CD38 cell expression was documented on lymphocytes of patients with a TTM score  $\leq 9$  as compared to those with higher TTM score. Similarly, CD38 mean fluorescence intensity (mfi) was differently distributed among Rai stages. Both the percentage and the mfi values corresponding to the 25th, 50th, and 75th percentiles were calculated. After giving a score of 0, 1, 2 and 3 for each percentile, a risk model was designed based on both percentage and mfi values, by summing individual scores. A favorable prognostic group (CD38 score  $\leq 3$ ) and a high risk group (CD38 score  $> 3$ ) were identified. Af-

ter a median follow-up of 36.5 months (range 0.17-214.2), 32 out of 161 patients died (19.9%). Patients with a CD38 score  $\leq$  3 had a significantly longer survival as compared with patients with a CD38 score  $>$  3 (percentage censored 86% and 71%,  $p=0.0026$ ). Age, sex, Rai and Binet stages, TTM score and bone marrow histology pattern entered a Cox regression multivariate analysis along with CD38 score. This latter remained the only variable with a significant impact on survival ( $p=0.0103$ ; RR 3.5, 95% C.I. 1.3-9.2).

### C039

#### VASCULAR ENDOTHELIAL GROWTH FACTOR LEVEL IS A MARKER OF DISEASE-PROGRESSION IN EARLY CLL

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Evidence for abnormal angiogenesis in the bone marrow (BM) of pts with CLL has been recently reported (Kini et al, Blood 1998,10:717a). However, clinical implications of such a finding are not completely understood. With this background we started with the present study specifically aimed at measuring with an enzyme linked immunosorbent assay (ELISA) serum levels of VEGF (Quantikine R, R & D System) in 68 CD5-positive B-cell CLL pts. Levels of S-VEGF ranged from 5.9 to 1190 pg/ml (median, 194.8 pg/ml). Although, a higher than the median value of S-VEGF was associated with a more advanced clinical stage ( $P = 0.01$ ). no correlation was found with serological markers representative of tumor mass such as LDH ( $P = 0.701$ ),  $\beta$ -2 m ( $P = 0.251$ ) and IL-6 ( $P = 0.331$ ). Observations in other types of cancer suggesting that increased levels of S-VEGF have a pivotal role in promoting progression of neoplastic disease, led us to investigate such an association in 42 CLL stage A pts. After a median follow-up time of 13 mo. (range, 2 to 40 mo.) 13 out of 42 (31.7%) pts progressed to a more advanced clinical stage (i.e., 7 to stage B and 6 to C); the risk of disease-progression (DP) being 31% at 24 mo. Pts whose S-VEGF serum levels were above the median value had an increased

risk of DP (median, 33 mo.) in comparison with those whose S-VEGF levels were below the median value (median not reached at 40 mo;  $P = 0.01$ , HR 0.235, 95% C.I. 0.084 to 0.773). Interestingly, characteristics of stage A pts stratified according to median level of S-VEGF were alike with respect to main prognostic features such as Rai substage ( $P=0.08$ ), absolute PB lymphocytosis ( $P=0.368$ ), LDT ( $P=0.870$ ), BM histology ( $P=0.952$ )  $\beta$ -2 m ( $P=0.128$ ). Finally, elevated levels of S-VEGF added prognostic information to the subclassification of stage A. Median time of PFS was 6 mo. for pts belonging to non-smoldering CLL and S-VEGF  $>$  median value while it was not reached at 40 mo. by pts with smoldering + non-smoldering with S-VEGF  $<$  median value ( $P<0.0001$ ). The results demonstrate that S-VEGF adds prognostic information to the definitions of smoldering and non-smoldering CLL.

### C040

#### MULTICENTRE PROSPECTIVE RANDOMISED TRIAL OF FLUDARABINE VERSUS CHLORAMBUCIL AND PREDNISONE IN PREVIOUSLY UNTREATED PATIENTS WITH ACTIVE B-CHRONIC LYMPHOCYTIC LEUKAEMIA (B-CLL): FINAL REPORT

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Fludarabine (FLU) is a promising new drug for the treatment of B-CLL. A randomised prospective multicentre study began in October 1994 to compare response rate and safety of FLU 25 mg/sm by 1/2 hr i.v. infusion daily for 5 consecutive days q 4 weeks, versus standard therapy with Chlorambucil (CHL) 30 mg/sm orally on days 1 and 15 plus Prednisone (P) 40 mg/sm i.m. on day 1 to 5 and 15 to 19 q 4 weeks. Previously untreated patients, with active B-CLL, RAI intermediate or high risk stages, entered the study. Patients receiving minimum 6 courses of chemotherapy were evaluated for response after the 6th course. Patients in CR received two further courses of chemotherapy, patients in PR received a 3 fur-

ther courses. Patients with progressive (PD) or stable disease (SD) after 3 and 6 courses of chemotherapy respectively, stopped treatment and were evaluated for survival. 150 eligible patients entered the trial: 75 were randomised to receive FLU and 75 CHL+P. 142 patients are valuable for response (NCI criteria), 69 in FLU arm and 73 in CHL+P arm. Response rate (CR+PR) was 71% (46+25) in FLU arm and 71% (37+34) in CHL+P arm. Refractory CLL (SD+PD) were 19% (10+9) and 18% (11+7) respectively. Toxicity was comparable in the two treatment groups. Response Duration (RD) is longer in the FLU arm (28 mo. vs. 21;  $p=0,007$ ). Our results confirm the high effectiveness of FLU in the treatment of untreated CLL: CR rate is faster and more frequent compared with CHL+P, but further investigations need to confirm the results on survival.

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#### **C041 CLINICAL AND BIOLOGICAL RESULTS ON 20 CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) PATIENTS (PTS) AFTER UNMANIPULATED PERIPHERAL BLOOD STEM CELL TRANSPLANT (PBSCT)**

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As of January 1995, 20 high-risk CLL pts, median age 46.5 (21-58), in complete remission (CR) after fludarabine, were offered a PBSC collection and reinfusion program following high-dose chemotherapy. Due to unsatisfactory PBSC collection, 4 pts received bone marrow cells. Sixteen pts received PBSC with a median number of CD34+ cells of  $3.55 (1.5-11.3) \times 10^6/\text{Kg}$ . All pts engrafted: median time to neutrophils  $>0.5 \times 10^9/\text{L}$  and to platelets  $>20 \times 10^9/\text{L}$  was 12 and 15 days respectively. One pt died early (2 months) after autograft for infection. Fourteen on 19 evaluable pts showed during the post-transplant follow-up a molecular remission, 3 of them presented a molecular relapse at 16, 24 and 28 months from PBSCT, being actually in

clinical CR 8 (2 pts) and 13 (1 pt) months from molecular relapse. Clonality was persistently observed at molecular level in 5 pts, 1 of them died for secondary neoplasia 17 months after PBSCT, 2 showed a clinical relapse at 26 and 33 months from transplant and 2 pts remain in clinical CR at 6 and 15 months from PBSCT. The projected disease free survival probability is 0.54 ( $\pm 0.2$ ) at 44 months from transplant, while the overall survival probability is 0.85 ( $\pm 0.5$ ). After PBSCT, immunological analysis showed a persistent inversion of CD4/CD8 ratio and marked decrease of total peripheral CD4+ cells. At 12 months from transplant, the absolute median number of circulating CD4+ cells/ $\mu\text{l}$  was 325 (4-648) for the 12 evaluable pts and, at 24 months, 410 (40-792) for the 8 evaluable pts. Our data suggest that, despite the use of unmanipulated PBSC, autograft could be useful in prolong molecular and clinical remissions in high-risk CLL pts responding to fludarabine therapy. The long-lasting impairment of immune repertoire after transplant has to be taken in account in the pts management.

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#### **C042 NEW TREATMENT APPROACH FOR REFRACTORY OR RELAPSED CHRONIC LYMPHOCYTIC LEUKEMIA: PRELIMINARY RESULTS WITH ANTI-CD20 MABTHERA**

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Preliminary trials in relapsed low-grade lymphoma have shown that chimeric anti-CD20 monoclonal antibody Rituximab (MabThera) is a new therapeutic option for CD20+ B-cell lymphoma. So far, most studies have been performed in recurrent follicular forms; less is known on MabThera activity in other low-grade subtypes and chronic lymphocytic leukemia (B-CLL). Here we report preliminary results on MabThera efficacy in 7 patients with refractory or relapsed B-CLL. All patients had CD20+ B-CLL; they were either in second or further relapse or refractory to initial therapy; 4 of them presented with very high lymphocyte counts (up to  $280.000/\mu\text{L}$ ), low gam-

maglobulin levels and history of recurrent infections; 2 had autoimmune anemia and/or thrombocytopenia. MabThera was given at 375 mg/m<sup>2</sup> over 8-hour i.v. infusion, for 4 consecutive weekly administrations. All patients received adequate i.v. hydration. MabThera was well tolerated; chills and fever often developed during the first infusion, but subsided following steroids. No severe complications occurred. A rapid and sustained peripheral B-cell decrease was observed in all patients, with lowest values reached within few hours after MabThera infusion. A striking lymphocyte count reduction was observed in the 4 patients presenting with massive peripheral blood involvement: their median lymphocyte count dropped from 88.000/mL to 3.300/mL after 4 MabThera courses; a variable extent of response could be documented also in bone marrow, lymph nodes and spleen; by contrast, minor responses were observed in the 2 patients with autoimmune anemia and thrombocytopenia. This preliminary experience indicates that MabThera may be an effective therapeutic option also for B-CLL. It allows to avoid the use of aggressive cytoreduction. This makes MabThera particularly suitable for heavily pretreated B-CLL patients, who are at high risk of infection when managed with presently available chemotherapeutic drugs.

#### C043

### HCV INFECTION IN NHL: CLINICO-PATHOLOGICAL CORRELATIONS IN 260 CASES

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The association between Hepatitis C virus and B-lymphoproliferative disease (including NHL and mixed cryoglobulinaemia) has been reported in various studies, especially from Italy. The association between HCV and NHL has not been confirmed in Great Britain, whereas in Italy the prevalence of HCV positivity varies between 9% and 42%. In our series we evaluated the correlation between HCV infection and clinico-pathological features of associated lymphomas. **Design and Methods:** We retrospectively analyzed 260 patients with

overt-NHL (histological diagnosis was made according to REAL classification), all from our geographical area (north-east of Italy). Only patients without obvious risk factors for HCV infection were included in the study. We tested their serum for the presence of HCV antibodies (ELISA and RIBA) and most of the positive were also examined for the presence of HCV-RNA (by RT-PCR) and of cryoglobulins. We also evaluated 100 patients from the same area, with others onco-haematological disorders, as controls. **Results:** HCV antibodies were present in 48/260 (18,4%) NHL patients. The infection was documented before the diagnosis of NHL (1-8 yrs) in most of the positives. The prevalence of HCV infection in general population in Italy varies between 0,8% and 2,8%; in our control group it was 4%. Viral RNA was found in the serum of 93% (28/30), cryoglobulins in 78% (18/23) of tested patients. None of the 15 T-NHL cases was HCV+. Of note, marginal zone lymphomas (in particular non-gastrointestinal MALTomas) had the highest rate of HCV+ cases (33,3%) followed by lymphoplasmacytoid lymphomas (28%). Positive patients were also significantly characterized by a primitive extranodal localization (54% vs 28%, p<.005). Salivary glands, spleen, tonsils and liver were frequently involved. HCV+ patients were also significantly older than negatives (81% over 50 yrs vs 60%, p=.05). **Conclusions:** Our results confirm the importance of the association between HCV and B-NHL in our geographical area and suggest a correlation between the infection and particular histotypes and primitive localization of lymphomas.

#### C044

### RANDOMIZED TRIAL OF FLUDARABINE VERSUS FLUDARABINE AND IDARUBICIN AS FRONT-LINE TREATMENT IN PATIENTS WITH LOW- GRADE NON-HODGKIN'S LYMPHOMA

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From September 1995 to July 1998, 199 patients aged 25-65 (median 54) years with newly diagnosed stage II-IV LG-NHL, stan-

standard risk according to the International Prognostic Index (IPI), were enrolled in a multicenter, 1:1 randomized study. Of the 199 patients evaluable, 101 were allocated to the FLU arm (6 monthly cycles of FLU 25 mg/m<sup>2</sup>/day on day 1 thru 5), and 98 to the FLU-ID arm (6 monthly cycles of FLU 25 mg/m<sup>2</sup>/day on day 1 thru 3 and idarubicin 12 mg/m<sup>2</sup> on day 1). On the FLU arm, CR and PR rates were 47% and 37% respectively, while on the FLU-ID arm, they were 39% and 42% respectively. In-depth analysis of the CR rate with respect to histologic type showed that FLU treatment appeared to be superior to FLU-ID against follicular lymphomas (60% vs. 40%), while FLU-ID was more effective against non-follicular lymphomas (small lymphocytic 43% vs. 29%, immunocytoma 38% vs. 23%, respectively). No striking differences were observed between the two protocols in terms of overall response or toxicity, which was generally mild. However, with a median follow-up of 19 months, only 29 (62%) patients who received FLU alone have maintained their first CR, as compared to 32 (84%) of those who received FLU-ID therapy. Although the FLU-ID regimen may not significantly improve the induction of CR in most LG-NHL patients, our preliminary data do suggest that with respect to FLU alone it may be capable of conferring a longer-lasting CR and that it is superior in terms of CR rate in small lymphocytic and immunocytoma subtypes.

#### **C045 STUDY OF THE IMMUNOSUPPRESSION AFTER FLUDARABINE, CYCLOPHOSPHAMIDE AND DEXAMETHASONE (FLUCYD) IN INDOLENT LYMPHOMA**

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We monitored post-treatment immunosuppressive toxicity of the Flucyd combination in 24 pts with advanced low-grade NHL (21 pts) or CLL (3 pts). Median interval from diagnosis: 44 mos (7-126); median no. of previous chemotherapies: 2 (1-4). No patient

had transformed lymphoma or CLL-Richter. Treatment: fludarabine 25 mg/m<sup>2</sup>/day + cyclophosphamide 350 mg/m<sup>2</sup>/day + dexamethasone 20 mg/day in 3-day courses repeated every 4 weeks. Twenty two pts received 5-6 courses and 2 pts 3-4 courses. The overall response rate was 79% (8 CR, 11 PR, 5 failures); 11 pts relapsed or progressed (3 to 19 mos from response); eight pts are still in CR or PR 3 to 27 months from response. The CD4+ and CD8+ lymphocyte counts decreased during therapy. In 19 responders monitored off-therapy at 3, 6, 9, 12 months after Flucyd or until relapse/progression, CD4+ and CD8+ counts were persistently low with minimal recovery over time:

	CD4+ / $\mu$ L		CD8+ / $\mu$ L	
	Median	Range	Median	Range
Pre-treatment	484	142-1865	520	82-2372
After three courses	260	71-912	394	129-2000
Final	198	71-637	399	90-3000
3 months F-U (N = 19)	202	96-705	440	145-3300
6 months F-U (N = 13)	205	105-604	460	263-1791
9 months F-U (N = 12)	252	155-818	462	150-1500
12 months F-U (N = 8)	229	135-466	465	240-1200

During treatment, 16 infectious episodes occurred in 11 pts. No delayed opportunistic infections occurred in responders while off therapy. Five pts evolved into high-grade B-cell NHL and in 1 pt transformation was highly probable on clinical grounds. The incidence of transformation (25%) was not higher than expected. In conclusion, fludarabine combined with cyclophosphamide and dexamethasone is effective therapy for indolent lymphoma. This combination produces prolonged T-lymphocytopenia. However, T-cell dysfunction in patients achieving response is not associated with higher incidence of infections and does not influence clinical outcome.

## **AUTOLOGOUS TRANSPLANTATION**

**C046**

### **HUMAN HERPESVIRUS-8 (HHV-8) VIREMIA OCCURS IN AUTOLOGOUS PERIPHERAL BLOOD STEM CELL (PBSC) TRANSPLANT PATIENTS AND IS ASSOCIATED WITH CLINICO- PATHOLOGIC MANIFESTATIONS OTHER THAN KAPOSI'S SARCOMA (KS)**

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We used polymerase chain reaction (PCR) with degenerate primers targeting the DNA polymerase gene of herpesviruses, to investigate the presence of herpesviral sequences in the sera collected from 14 autologous PBSC transplant patients, showing at least one pathologic event after transplantation, in absence of a documented bacterial, fungal or viral (other than herpes) infection. During this survey, we documented the early occurrence of HHV-8 viremia in two lymphoma patients, undergoing unselected PBSC transplantation and receiving immunoglobulin and i.v. acyclovir prophylaxis. Using PCR with primers specific for two different regions of HHV-8 genome (ORF 26 and K1), we detected HHV-8 sequences in the sera collected immediately before and/or concomitant with clinical events in both patients. HHV-8 genomes were classified on the basis of the sequencing of the hypervariable K1 region, as variant A and variant C respectively. Serum HHV-8 DNA was no longer detectable after the disappearance of the clinical symptoms. Clinical events associated with the detection of HHV-8 viremia were: fever, cutaneous rash, diarrhoea, elevated aminotransferases, in one patient (at day +12) and fever and bone marrow (BM) failure in another patient (at day +62). Of note, the specific HHV-8 latent transcript (T0.7) could be detected in stromal cells in the aplastic

bone marrow from the latter patient, by in situ hybridization. Both patients had antibodies to HHV-8 antigens, as detected by a lytic immunofluorescence assay (IFA) before transplantation so that active HHV-8 infection probably reflects viral reactivation. HHV-8 infection is associated with an increased risk of post-transplant KS, in recipients of kidney allografts, but KS is exceptional in the setting of autologous and allogeneic BMT patients. Our study shows, for the first time, that HHV-8 viremia may occur, in absence of KS, also in the setting of autologous PBSC transplantation, at least in our geographical area (the lower Po valley, Northern Italy), where HHV-8 seroprevalence in the blood donor population is about 13%. HHV-8 may be considered in the differential diagnosis of the possible causes of graft failure in the setting of autologous transplantation.

**C047**

### **EFFECTS OF HIGH DOSE CHEMOTHERAPY GIVEN WITH G-CSF ON THE HEMOSTATIC SYSTEM ACTIVATION IN PATIENTS WITH BREAST CANCER UNDERGOING AUTOLOGOUS TRANSPLANTATION**

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Chemotherapy and hematopoietic growth factors increment the risk of thrombosis in malignancy. New protocols for breast cancer therapy include autologous transplantation of CD34+ hematopoietic progenitor cells (HPC) mobilized in peripheral blood by high dose cyclophosphamide (CTX, 7g/mq) for 1 day followed by G-CSF (5µg/kg/d). We have previously observed that G-CSF administered to healthy donors of HPC induces a transient hypercoagulable state (Blood, 1999). To verify whether CTX has additional effects on the hemostatic system activation induced by G-CSF, we have studied 18 consecutive patients with stage II breast cancer receiving CTX and G-CSF for HPC mobilization. Plasma markers of hypercoagulation (TAT, F1+2, D-dimer), endothelial (TM, vWF, t-PA and PAI-1) and leukocyte activation (elastase, myeloperoxidase [MPO]) were determined at the

following time intervals: 1. basal (T0), 2. after CTX, before starting G-CSF (T1), and 3. at the end of G-CSF, before HPC apheresis (T2). Results were compared to those obtained in 26 consecutive HPC healthy donors receiving G-CSF alone (10µg/kg/d). At baseline (T0), the plasma levels of hypercoagulation markers of the patients were significantly greater than those of healthy donors ( $p < 0.05$ ), endo-thelial and leukocyte parameters being not different. In T1, after CTX, patient endothelial markers significantly increased compared to T0 (T0 vs T1: TM:  $31.7 \pm 4.3$  vs  $38.2 \pm 4.3$  ng/ml; vWF:  $120 \pm 8$  % vs  $164 \pm 20$  %, t-PA:  $2.5 \pm 1.7$  vs  $11.8 \pm 3.6$  ng/ml; PAI-1:  $43.2 \pm 6.5$  vs  $113.4 \pm 28.2$  ng/ml). Among hypercoagulation markers only F1+2 increased significantly (T0 vs T1:  $1.49 \pm 0.12$  vs  $2.03 \pm 0.37$ ;  $p < 0.05$ ). In addition also the leukocyte activation markers augmented (T0 vs T1; elastase:  $75.6 \pm 8.7$  vs  $221 \pm 57$  µg/L; MPO:  $10.2 \pm 2$  vs  $234 \pm 35$  ng/ml). At the end of G-CSF treatment (T2), before leukapheresis, the patients' vWF, elastase and MPO levels further increased compared to T1 ( $p < 0.05$ ), while t-PA and PAI-1 significantly decreased. All the other parameters stayed unmodified. Furthermore in T2, the patient group showed plasma levels of TAT, D-dimer and vWF significantly greater than those of healthy donors after G-CSF ( $p < 0.01$ ). This study shows that high dose CTX significantly affects the hemostatic system, in particular the endothelial compartment. The combination CTX + G-CSF worsens this effect. Prospective large clinical studies are needed to evaluate the clinical utility of these plasma markers to predict the risk of thrombosis and post-transplant vascular complications in these patients.

## CO48

### HIGH DOSE MITOXANTRONE AND L-PAM FOLLOWED BY PBPC AUTOGRAFT: HIGH TOLERABILITY IN SPITE OF LOW AND PARTIALLY REVERSIBLE CARDIOTOXICITY

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The use of peripheral blood progenitor cells (PBPC) has minimized hematologic toxicity of autograft procedures; studies are now addressed to improve also extrahematologic tolerability. In this view we have evaluated a new conditioning regimen including mitoxantrone (60 mg/sqm at day -5) and melphalan (L-PAM) (180 mg/sqm at day -2) (Mitox/L-PAM). Autograft was the conclusive phase of intensified programmes with high-dose sequential chemotherapy. Mitox/L-PAM was evaluated in 112 patients (102 non-Hodgkin's lymphomas and 10 Hodgkin's Diseases). Their median age was 44 years (range 16-61), M/F ratio was 64/48; 24 patients had relapsed disease. A slight, transient increase of serum bilirubin, liver function tests and CPK levels was observed in a minority of patients; no patient showed acute signs of heart or liver failure. Prompt post-graft hemopoietic recovery was observed in most patients. Consolidation radiotherapy (RT) could be delivered to 63 out of 69 candidate patients, within 6 mos. since autograft. Eight patients needed additional PBPC reinfusion following RT; in spite of that, one of these patients died for sepsis due to persistent pancytopenia. This was the single fatal toxic event in this series of 112 patients. No late graft failure was observed ever, although a certain stem and progenitor cell pool reduction could be consistently documented. Left ventricular (LV) function was assessed by multigated radionuclide scan. LV ejection fraction (EF) was significantly reduced at a post graft control (median 46.5% vs. 55%;  $p < 0.0001$ , compared to pre graft value) in 44 evaluated patients. However a partial recovery could be documented in 34 evaluable pts. after 1 year or later since autograft (median LV-EF 48% vs 45%;  $p < 0.05$ , compared to LV-EF evaluated

within 6 mos). So far, 90 pts. are alive, with a projected overall survival of 78% at 7 yrs., at a median follow up of 3.5 yrs.. We conclude that PBPC autograft after Mitox/L-PAM is well tolerated at both early and late follow-up and implies low and reversible cardiotoxicity.

#### **C049**

### **IMMUNE RECONSTITUTION AFTER AUTOLOGOUS PERIPHERAL BLOOD STEM CELL TRANSPLANTATION**

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We studied the phenotypic immunologic reconstitution of different subsets of T, B and NK cells after autologous peripheral blood stem cell transplantation (PBSCT) within the first 3 months following transplantation. The recipients were 36 consecutive adults (median age 45 years, range 21-62), who had received autologous PBSCT for multiple myeloma (N.=14), high-grade non-Hodgkin's lymphoma (N.=13), Hodgkin's disease (N.=7), chronic myeloid leukemia (N.=2); 49 healthy subjects age-matched were also studied as control group. In transplant recipients, tests were performed before transplant and at 30, 60, 90 days after bone marrow take. Peripheral blood lymphocyte subsets were studied by direct immunofluorescence and flow cytometry, using a FACS (FACSort, Becton Dickinson) and a lyse-no-wash sample processing of total peripheral blood. We demonstrate in this study that: 1) the recovery of CD3+ T-cells is prompt, within the first month post-transplantation, and appears mainly due to early and faster reconstitution of CD3+/CD8+ T cells, reaching supranormal levels and resulting in an inverted CD4/CD8 ratio during the study period; 2) the reconstitution of CD3+ T-cells shows a marked increase in activated HLA-DR+/CD3+ and CD8+/CD57+ lymphocytes by 60 days after bone marrow take; 3) the recovery of the CD4+ T-cells is markedly delayed during the first three months after transplantation, with a predominance of memory-type CD29+/CD4+ T-cells, which

are in the normal range during the study period, and a relative absence of naïve-type CD45RA+/CD4+ T-cells; 4) an early and durable overshoot of CD5+/CD19+ B-cells above normal levels is observed during the study period, whereas CD20+ B-cells reconstitution is reached at three months; 5) CD16+/CD56+/CD3- NK lymphocytes show a faster recovery with a significant increase by 60 days following PBSCT. These data on the kinetics of post grafting immune reconstitution may have biological and clinical relevance.

#### **C050**

### **FACTORS AFFECTING VIRAL INFECTIONS AFTER AUTOLOGOUS PERIPHERAL BLOOD PROGENITOR CELL TRANSPLANT**

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Viral infections that occur 100 days after bone marrow transplantation (BMT) are mostly due to cytomegalovirus (CMV) and varicella zoster virus (VZV), the incidence of the later is around 30% in allogenic BMT and around 20% in autologous BMT. Very few reports analyzed viral infections after autologous peripheral blood progenitor cell transplant (PBPCT) and the largest series reported a frequency ranging from 6 to 31%. It is possible that the recovery pattern of circulating T or NK cells play a critical role in the development of viral infections. Our study included 164 patients (median age 47 years, range 16-68; M/F=84/80) affected by lymphoid malignancies (77 NHL, 19 HD, 35 MM and 5 LLA), solid tumors (16) and myeloid malignancies (9 AML, 3 CML). Before PBPCT 53 patients (31%) were in complete remission, 93 (57%) in partial remission and 20 (12%) with disease progression. All patients had a good performance status although a lot of them were heavily pretreated (79 patients had been received > 2 chemotherapy regimens, 85 a number of chemotherapy courses > 10). PBPCT included high dose Melphalan (140-200 mg/mq) administered alone or in combination in most patients (70%) and stem cell support, with a me-

dian of CD34+ cells reinfused of  $6 \times 10^6/\text{kg}$  (range 0.51-51.3). Seventy patients (43%) received a steroid dose higher than 180 mg during or following PBPCT. Flow cytometric analysis of the lymphocyte subsets was performed at 1, 3, 6, 9, 12 months in the first year following the transplant and every 6 months in the subsequent years. We therefore investigated the viral infections occurring 30 days after PBPCT. Twenty-nine patients (17,7%) developed viral infection (1 (0.6%) CMV and 28 (17%) VZV). Fatal CMV infection occurred 35 days after transplant whereas the median onset of VZV infections was 10 months after transplant (range 1.2-18.8 months). The actuarial risk of VZV was 12% at 12 months and 18% at 24 months. Twenty-eight patients had metamer herpes zoster and only one patient developed varicella. The median CD4+ cells count remained  $< 400/\mu\text{l}$  until 12 months following PBPCT (1<sup>th</sup> month= $249/\mu\text{l}$ ; 3<sup>th</sup> month= $260/\mu\text{l}$ ; 6<sup>th</sup> month= $287/\mu\text{l}$ ; 9<sup>th</sup> month= $245/\mu\text{l}$ ; 12<sup>th</sup> month= $383/\mu\text{l}$ ). The actuarial probability of achieving 400 CD4+ cells resulted of 30% at 3 months, of 50% at 6 months and of 55% at 12 months. Univariate analysis showed that age, diagnosis, previous chemotherapy, dose of steroid administered, CD34+ cells reinfused and lymphocyte count at 1 month ( $400 < \text{CD4+} \leq 400$ ) were risk factors associated with viral infections. Multivariate analysis selected three factors: CD34+ cells reinfused ( $p=0.03$ ; OR= 1.8), dose of steroid ( $p=0.01$ ; OR=1.7) and CD4+ cells ( $p=0.03$ ; OR=1.7). Moreover, the probability of achieving  $400/\mu\text{l}$  CD4+ cells was significantly lower in patients who developed viral infections than in patients who did not. In our series the crude incidence of VZV infections was quite similar to that reported after autologous BMT and lower than that after allogenic BMT. All patients with VZV infection had a self-limited illness. We observed that the factors affecting development of viral infections depend directly (CD4+ cells count) or indirectly (dose of steroid administered and CD34+ cells reinfused) on the immunologic reconstitution after PBPCT.

## C051

### REDUCED LTC-IC FREQUENCY AND STROMA FORMATION IN BONE MARROW HARVESTS OF PATIENTS WITH ACUTE MYELOBLASTIC LEUKEMIA (AML) IN REMISSION

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A prolonged aplasia was observed after autologous bone marrow transplantation (ABMT) in patients with AML. In this study, the cellularity, CD34 and CFU-GM were evaluated in the marrow harvests of 15 adult patients with AML in first complete remission (CR) after an induction and consolidation program according to the EORTC/GIMEMA AML10 protocol. Patients underwent ABMT after conditioning with the BU/CY2 combination. After thawing of the cryopreserved marrow, the LTC-IC frequency was evaluated by limiting dilution analysis. The in vitro confluence of stromal cells was also evaluated and expressed as the percentage of the flask surface covered by stroma at 5 weeks of culture. The bone marrow from 10 volunteer donors was used as normal controls for LTC-IC frequency. A marked reduction in LTC-IC frequency was observed in the marrows of patients in contrast to normal controls (mean  $1.65$  vs.  $9.85 \times 10^{-5}$ ;  $p=0.0001$ ). In vitro stroma formation was impaired in the majority of cases: after 5 weeks of culture no growth was observed in one patient, a poor confluence ( $<60\%$ ) in 8 cases, a fair confluence ( $\geq 60\%$ ) in 5 cases and a complete confluence (100%) only in one case. The median duration of granulocyte ( $N < 500$ ) aplasia was 42 days (range 18-301) and of platelet ( $pt. < 20.000$ ) aplasia was 65 days (range 15-340). The patients with a neutropenia shorter than the mean showed a higher cellularity of the harvest ( $4.39$  vs.  $2.40 \times 10^8/\text{kg}$ ;  $p=0.02$ ) and a higher mean LTC-IC frequency ( $2.63$  vs.  $0.81 \times 10^{-5}$ ;  $p=0.02$ ). No significant difference was observed for CD34 (mean  $3.28$  vs.  $10.13 \times 10^6/\text{kg}$ ;  $p=0.16$ ) and CFU-GM (mean  $8.41$  vs.  $8.97 \times 10^4/\text{kg}$ ;  $p=0.1$ ). The length of thrombocytopenia was not influenced by any of the considered variables. No correlation was observed by in vitro

stroma formation and aplasia duration. Our data would confirm the reduced hemopoietic capacity and the impaired microenvironment in the patients with AML in remission. This marrow damage may be disease-related or induced by the aggressive chemotherapy regimens used in induction and consolidation.

## C052

### SELECTION AND TRANSPLANTATION OF AUTOLOGOUS CD34+B-LINEAGE NEGATIVE CELLS IN ADVANCE PHASE MULTIPLE MYELOMA (MM) PATIENTS: A PILOT STUDY

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In this study we evaluated the feasibility of sequential positive and negative selection of stem cells to achieve tumor-free autografts in MM. Moreover, we assessed the safety and the hematopoietic efficacy of the transplantation of doubly selected (CD34+B-lineage negative) autologous cells. Fourteen MM patients with advanced disease had their PBSC mobilized with cyclophosphamide (Cy; 7 gr/m<sup>2</sup>) and G-CSF. CD34+ cells were enriched in 12 of the patients by the avidin-biotin immuno-absorption technique. Subsequently, CD10+, CD19+, CD20+ and CD56+ cells (B-lin cells) were removed by immunomagnetic depletion. Minimal residual disease (MRD) was detected by flow cytometry and polymerase chain reaction (PCR) based molecular analysis of the patient specific IgH complementary-determining region III (CDRIII). All the patients were reinfused with CD34+B-lin negative cells after administration of high-dose melphalan (Me; 200 mg/m<sup>2</sup>). Positive/negative selection of stem cells resulted in a median recovery of 33.3% of the initial content of CD34+ cells (range 13.4-67%) with a median purity of 72.7% (40-97.6%). All the evaluable patients had detectable disease in PBSC collections. Molecular assessment showed the persistence of myeloma cells in 6/7 cases after

the first step of positive selection of CD34+ cells. However, molecular evaluation of IgH CDRIII region showed the disappearance of tumor cells in 6/7 patients following negative depletion of B-cells. Twelve patients received a median of 3.9 x 10<sup>6</sup> CD34+B-lin negative cells/Kg (range 1.2-6.5) and showed a rapid reconstitution of hematopoiesis. The median time to an absolute neutrophil count (ANC)  $\geq$  0.5 x 10<sup>9</sup> /L was 12 days and to 20 and 50 x 10<sup>9</sup> platelet/L 14.5 and 19.5 days, respectively. The median time to hospital discharge after reinfusion was 15.5 days. These results were superimposable with those of two similar cohorts of patients who received either unmanipulated PBSC or positively selected CD34+ cells after the same conditioning regimen. No late infections were observed. We conclude that autotransplantation of purified CD34+ B-lin negative cells is associated with a rapid and sustained recovery of hematopoiesis and low peritransplant morbidity. Sequential positive and negative enrichment of stem cells may reduce tumor cell contamination in B-cell malignancies below the lower limit of detection of molecular analysis.

## C053

### A NEW EFFECTIVE PURGING TECHNIQUE FOR AUTOLOGOUS TRANSPLANTATION IN MULTIPLE MYELOMA

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To reduce the tumor cell contamination of G-CSF mobilized peripheral blood circulating progenitor cells (CPC), we developed a two-step negative selection procedure whereby CPC can be effectively purged of contaminating neoplastic cells by magnetic microbeads and a SuperMACS separator (Miltenyi Biotech, Germany) (Rambaldi et al., Blood 1998). We applied this purging technique to Multiple Myeloma patients using anti CD19, CD56, CD10 and CD138 microbeads for in vitro purging. Thirity-four newly diagnosed MM patients received 3 cycles VAD followed by Cyclophosphamide (CTX, 7 gr/sqm) + G-CSF (5 mcg/kg/day) for stem cell collection. Thereafter they were randomized to receive autologous unmani-

pulated CPC (Arm A, 18 patients) versus highly purified plasma cell-purged (Arm B, 16 patients) to support tandem sequential transplants (TRX) conditioned with Melphalan (200 mg/sqm) and Melphalan (140 mg/sqm) plus Total Body Irradiation (TBI, 1200 cGy) for the second transplant. Aims of this study were to evaluate: a) the efficacy of in vitro purging on the neoplastic plasma cell fraction, b) the quality of the hematopoietic and lymphoid reconstitution after transplantation. By immunophenotype and PCR analysis performed with consensus oligonucleotide primers for the CDR3 region of rearranged heavy chain alleles we can demonstrate that in all cases the unmanipulated apheretic products contained a heavy plasma cell contamination as opposed to the purified stem cell fraction obtained after in vitro purging which showed a remarkable ( $\geq$  three logs) reduction of tumor cell contamination. Two apheresis were sufficient to meet the required minimum criteria of  $5 \times 10^6$  CD34+ cells/kg to support each transplant and to have a back-up source of unmanipulated stem cells. The hematologic engraftment was rapid and not different in the two arms. The immunologic reconstitution (as determined by enumeration of T, B and NK cells) was comparable in both arms and no transplant related mortality was seen so far. These results suggest the lack of any significant hematologic and immunologic toxicity associated with transplantation of plasma cell-purged CPC. The clinical benefit of this procedure still remains to be determined.

## C054

### PBPC AUTOTRANSPLANTATION IN ELDERLY PATIENTS: A SINGLE CENTER EXPERIENCE

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Between May 1994 to November 1998 we enrolled 25 consecutive patients (age > 60 years) for PBPC autologous transplantation. The age ranged between 60 to 67 years

(median 63); 12 were female and 13 male; 11 patients were affected by Multiple Myeloma (MM), 5 High-Grade non Hodgkin Lymphoma, 4 Low-Grade non Hodgkin Lymphoma, 3 Acute Myeloid Leukemia, 1 Chronic Lymphocytic Leukemia and 1 Breast Cancer; the performance status (WHO) was 0-1. Six patients were in first complete remission (CR), 19 patients received salvage therapy after failure (11 relapse, 7 partial remission and 1 progression disease) of first-line chemotherapy. All patients received chemotherapy + G-CSF for PBPC mobilization, leucafereses were performed when the circulating CD34+ cell count was  $\bullet 20/\mu\text{l}$  starting on day + 13 as average (range: 11-18). After salvage chemotherapy, 5 patients obtained CR, 12 a partial remission (PR) and 2 had progression of disease (PD). A median of  $6 (3-14) \times 10^6/\text{kg}$  CD34+ cells and  $48 (20-315) \times 10^4/\text{kg}$  CFU-GM were collected. Patients received high-dose therapy consisting in Melphalan (6 cases), Busulfan-Melphalan (6 cases), Mitoxantron-Melphalan (5 cases), Thiotepa-Melphalan (2 cases), Busulfan-Cyclophosphamide (3 cases), BEAM (1 case), TBI or TBI + Melphalan (1 case respectively). Four patients affected by MM underwent a second autotransplantation with Busulfan-Melphalan as conditioning regimen. We observed 1 toxic death during the first 100 days for interstitial pneumonia; the engraftment was rapid and complete in all patients; the hemopoietic reconstitution was characterized by 11 (range: 8-15) days to reach neutrophils  $\bullet 500/\mu\text{l}$ ; 13 (range: 10-83) days to reach platelets  $\bullet 20,000/\mu\text{l}$  and 17 (range: 11-60) days to reach platelets  $\bullet 50,000/\mu\text{l}$ ; patients were generally discharged 15 days after the PBPC reinfusion. After transplantation 18 patients are in CR, 5 are in PR and 1 showed PD; at present 16 patients are alive (9 in CR, 4 in PR and 3 in relapse) and 8 died for PD with a median follow-up of 14 months (4-50). In conclusion in our experience the PBPC mobilization and transplantation is feasible in patients with age > 60 years and the toxicity of this procedure is acceptable with an early transplant related mortality of 4%.

## MYELODISPLASIA AND CHRONIC MYELOCYTIC LEUKEMIA

### C055 CYTARABINE INCREASES KARYOTYPIC RESPONSE AND SURVIVAL IN ALPHA- IFN TREATED CHRONIC MYELOID LEUKEMIA PATIENTS: RESULTS OF A NATIONAL PROSPECTIVE RANDOMIZED TRIAL

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Between Feb. 1994 and March 1997 the ICSG on CML recruited 837 newly diagnosed CML pts: 540 eligible pts with Ph<sup>+</sup> and/or bcr/abl<sup>+</sup> CML in 1<sup>st</sup> CP were randomized to IFN alone (265 pts) at a starting dose of 3.10<sup>6</sup> IU/d, increased to 9.10<sup>6</sup> IU/d from the 14<sup>th</sup> day onward or IFN + 10 days monthly courses of cytarabine, 40 mg/kg/d s.c. (275 pts). The endpoints were: hematological response (HR) at 6 mos, karyotypic response (KR) and overall survival. The analyses were performed as to April, 1999 when the mean observation period was 24 months (1-42). At 6 months 80% of IFN arm pts at risk got a HR as compared with 87% of IFN+LDAC pts; The best KR to date is presented below

Best KR	IFN (n.265) N. (% of random.)	IFN+LDAC (n.275) N. (% of random.)
KR absent (0% Ph <sup>-</sup> )	55 (20)	50 (18)
KR minimal (1-32% Ph-neg)	42 (16)	32 (12)
KR minor (33-65% Ph-neg)	31 (12)	39 (14) 39 (14)v
KR major (66-99% Ph-neg)	31 (12)	39 (14)
KR complete (100% Ph-neg)	19 (7)	38 (14)
Total evaluated	175 (67)	205 (72)

The rate of major+complete KR is significantly higher for the IFN+LDAC arm (77/275, 28%) with respect to IFN arm (50/265, 19%) (p = 0.01). It's noteworthy the double proportion of complete KR among IFN+LDAC pts. With a median observation

period of 24 months, 3 yrs survival is 85% for IFN+LDAC and 80% for IFN alone. Overall survival is significantly better for IFN+LDAC with respect to IFN alone (p = 0.03).

### C056 IN VITRO RESISTANCE OF CHRONIC MYELOID LEUKEMIA CFU-GM TO GROWTH FACTORS DEPRIVATION IS SUPPRESSED BY RETINOIDS ± α INTERFERON

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Bcr-abl fusion protein determines, in transfected murine cell lines, both resistance to growth factor deprivation and increased proliferative response to interleukin 3 (IL3). However, conflicting results have been reported about growth factor requirement by CFU-GM. We cultured progenitor-enriched (median value of CD34<sup>+</sup>:45%) CML cells, taken from patients with 100% Ph<sup>+</sup> bone marrow (BM) mitosis, in agar culture with scalar concentrations of IL3 or GM-CSF (0.02 – 20 ng/ml) for CFU-GM assay. Cells were also cultured in liquid medium (IMDM + 10% fetal bovine serum) without growth factor addition, for 7-11 days, starting from 5 x 10<sup>4</sup> cells/ml. In parallel cultures either all-trans (ATRA) or 13-cis retinoic acid (cRA) (5 x 10<sup>-7</sup> M) ± interferon α (IFN) (300 U/ml) were included in the medium. CFU-GM concentration was monitored at day 0, 4, 7 and 11 of culture. Identical experiments were performed with BM cells of control subjects. An increased response to both IL3 and GM-CSF was evidenced by CFU-GM from 1/2 patients, with 80% of maximal colony growth at 0.02 ng/ml (v.n.10-42%). Two more patients displayed analogous CFU-GM hypersensitivity to GM-CSF and one to IL-3 only. In liquid cultures normal CFU-GM from 7 BM samples decreased steadily (36±22% of day 0 value at the 7<sup>th</sup> day, 27±19% at day 11), whereas CML CFU-GM increased or remained unchanged in 13/16 cases and slightly decreased in 3 (average CFU-GM recovery of total cases: 120±46% at day 7, 117±45% at day 11). However, ATRA, cRA, and, par-

ticularly, ATRA+ IFN and cRA+IFN reduced CML CFU-GM recovery to normal values:  $58\pm 32$ ,  $60\pm 38$ ,  $30\pm 20$ ,  $33\pm 23\%$  respectively at the 7<sup>th</sup> day,  $28\pm 35$ ,  $29\pm 36$ ,  $18\pm 8$ ,  $7\pm 6\%$  at day 11. In conclusion, CFU-GM hypersensitivity to CSFs seems unfrequent, while resistance to growth factor deprivation is a common feature of CML CFU-GM. The association of either cRA or ATRA +  $\alpha$ IFN at therapeutical concentrations can completely abolish in vitro that survival advantage, bringing further evidence to a possible role of retinoids in combined treatment modalities.

### **C057**

#### **LIMITED NUMBER OF Ph-POSITIVE STEM CELLS SUSTAIN RELAPSE AFTER ALLOGENEIC BONE MARROW TRANSPLANTATION**

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We have performed cytogenetic analysis on bone marrow cells and on progenitor cell colonies in a patient who relapsed after allogeneic BMT for CML. She was subsequently treated with donor lymphocyte infusions (DLI) and achieved cytogenetic remission after 175 days from the first DLI. Two Philadelphia-positive clones were identified at relapse. One clone displayed an additional chromosomal abnormality:  $46,XX,t(3;11)(p21;p15),t(9;22)(q34;q11),del(13)(q14q34)$  probably induced by radio-chemotherapy in a single Ph-positive progenitor. This clone was able to sustain 20% of Ph-positive hemopoiesis for 5 months and therefore displaying the characteristics of a "stem cell". We would take this result to suggest that a limited number of leukemic stem cells are responsible of relapse after allogeneic BMT in patients with CML. This is also supported by the fact that long term culture initiating cells (LTC-IC) were all donor derived Ph-negative, whereas the majority of BM cells were Ph-positive. All together these data may be permissive of a high response rate to DLI and may also explain the slow pace of the disease at the relapse following BMT.

### **C058**

#### **LOW DOSE INTERFERON IN ESSENTIAL THROMBOCYTHAEMIA: REMISSION MAINTENANCE AND THROMBOSIS FREE SURVIVAL**

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In the period February 1994 - December 1996, 208 patients with Essential Thrombocythaemia (ET) were diagnosed, 42 out of these (20.2%) were selected for treatment with recombinant alpha-2b interferon (IFN) plus the antiaggregant indobufene. Major inclusion criteria were: no previous chemotherapy, age less than 60 years, a thrombo-haemorrhagic event and/or serious symptoms of disturbed microvascular circulation and/or platelets more than  $900.000/mm^3$ . The IFN dosage was 3 MU three times every week for two consecutive years. After this period, patients were randomly assigned to either stop IFN or to continue one more year with the weekly fixed single dose of 3 MU. At diagnosis 9 patients out of 42 had had a thrombotic event. At Jan 1999 the overall observation period was 111 years/patient. 34 patients out of 42 completed the 2 years of treatment: 21 patients had a complete response (CR,  $plts < 450 \times 10^3/mm^3$ ), 10 patients had a partial response (PR,  $450 < plts < 600 \times 10^3/mm^3$ ), 3 patients were non responder (NR,  $plts > 600 \times 10^3/mm^3$ ). 31 patients out of 34 were randomized after two years of treatment: no difference in the median platelet count was observed between the two groups after the randomization. Two thrombotic events and no bleeding were observed during the observation period. Prevalence of thrombotic events during the observation period was 1.5 %/year. 17 patients have a follow up period of more than 4 years: among these, 9 are still in CR or PR. IFN treatment was stopped in 8 patients: in 4 patients because of side effects, in 3 because of non response, and in one because of intercurrent thrombotic event. 31 (91%) out of the 34 patients treated for the two years period had a good haematological response (CR or PR). Moreover, outcoming data show a remission maintenance in 64%

of patients with a long follow-up. IFN plus indobufene allowed to obtain a good thrombosis-free survival and, in some patient, a long last-ing remission maintenance, also after IFN withdrawal.

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### **C059**

#### **LEUKEMIA AND CARCINOMA IN PATIENTS WITH ESSENTIAL THROMBOCYTHEMIA TREATED WITH HYDROXYUREA**

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Treatment with hydroxyurea (HU) is indicated in patients with essential thrombocythemia (ET) but retrospective studies have raised concern about the long-term leukemogenic risk of this agent. We updated a randomized clinical trial of HU vs. no chemotherapy that started in 1990 to examine the long-term effect of the drug, in particular the development of secondary malignancies. We randomized 114 patients with ET and age >60 years or previous thrombosis to HU (56 cases) or no cytoreductive therapy (NT, 58 cases). Two patients (1.7%) were lost to follow-up and 29 (50%) shifted from the NT group to the HU group during the observation period, mainly because of a thrombotic event. Patients were followed up for a median of 73 months. Analysis was by intention to treat. In the HU group, 46 of 54 patients (85%) are alive, compared with 49 of 58 patients (84%) in the control group (n.s.). Five patients (9%) in the HU group had thrombosis and 26 (45%) in the control group. Thrombosis-free survival was significantly different in the HU and control groups ( $p < 0.0001$ ). In the HU-treated arm, seven patients (13%) developed secondary acute leukemia, myelodysplastic syndromes or solid tumors, but only one control (1.7%). The difference in cancer-free survival between the two groups was also significant ( $p = 0.0321$ ). Multivariate logistic regression analysis identified previous therapy with busulfan as an independent predictor of cancer in HU-treated patients. In conclusion, HU therapy reduces the risk of thrombosis but increases the risk of secondary leukemia and carcinoma, particularly in ET

patients who have been treated with busulfan. The drug does not affect overall survival.

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### **C060**

#### **DIAGNOSTIC ROLE OF ENDOGENOUS ERYTHROID COLONIES (EEC) IN POLYCYTHEMIA VERA (PV) AS DEREGULATED EXPRESSION OF BCL-XL PROTO-ONCOGENE**

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Polycythemia Vera is a Myeloproliferative Chronic Syndrome (MCS) with a clonal disorder of stem cells which produces an increased number of erythroblasts in the bone marrow (BM) and erythrocytes in peripheral blood (PB). In the last years a number of new "positive" diagnostic criteria have been investigated besides those suggested by PVSG based on the exclusion of others MCS or reactive conditions. In PV, the in vitro presence of EEC is related to the expression of a EPO-independent clonal population of erythroblasts. Furthermore, in PV patients the BCL-XL has been identified, a proto-oncogene which inhibits apoptosis. The transcriptional deregulation of BCL-XL would release the erythroid progenitors from the normal mechanisms of differentiation's control thus promoting in vivo the increase of red-cell mass and in vitro the development of EEC. 148 patients with polyglobulia and 40 healthy controls were investigated in the study for the presence of EEC by in vitro clonogenic tests on BM and PB. Moreover, we searched by RT-PCR the BCL-XL transcript and the BCR-ABL rearrangement in the BM of 12 patients with PV, 10 with essential thrombocytosis (ET) and 7 healthy controls. 92 out of 148 patients (62%) had both diagnosis of PV (PVSG) and presence of EEC; 20/148 (14%) had presence of EEC but negative diagnosis of PV, in absence of secondary conditions; 18/148 (12%) had diagnosis of PV but absence of EEC; 18/148 (12%) were PV and EEC negative (secondary polyglobulia). In all controls EEC were absent. The BCL-XL was found in 8/12 patients with PV (83%) and 8/10 patients with ET (80%).

BCL-XL was negative in controls. BCR-ABL was negative in all subjects. In our study, presence of EEC (both in BM and PB) has a 100% specificity and 84% sensibility in diagnosing PV. Presence of EEC is especially useful in patients with absence of clinical criteria for PV. In ET, the EEC may be present but always together with the s-CFU-Mk. According to other authors, also in this study we found a low specificity of BCL-XL due to its presence in ET.

### **C061 NEOANGIOGENESIS CORRELATES WITH IN VITRO PATTERN OF GROWTH OF MYELODYSPLASTIC CELLS**

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The growth of new blood vessels from pre-existing vessels and capillaries, or neoangiogenesis, is crucial for tumor development. Recently Perez-Atayde suggested that angiogenesis can play a pivotal role also in leukemia. This prompted us to investigate the role of blood vessels generation in the pathogenesis and progression towards leukemia in myelodysplastic syndromes (MDS). With this aim we evaluated microvessel density (MVD) in bone marrow biopsies from MDS patients, healthy controls and subjects affected by infectious diseases (ID) and hyperplastic BM; MVD figures were then correlated with the in vitro pattern of growth (leukemic vs non leukemic according to Sawada et al) of myelodysplastic bone marrow mononuclear cells (BMMNC). **Patients and Methods:** 30 MDS patients, 14 normal controls and 5 patients with ID were enrolled. Bone marrow biopsies were immunostained with anti CD34 QBEnd/10 monoclonal antibody to evaluate (MVD) and hot spot, i.e. areas with the largest number of vessels (HS). BMMNC were obtained after gradient centrifugation and evaluated for CFU-GM in conventional agar assay. **Results:** MVD and HS were similar in controls and ID (respectively MVD  $6 \pm 2$  vs  $10 \pm 8$ ; HS  $13 \pm 4$  vs  $19 \pm 13$ ) and significantly higher in MDS (MVD  $21.5 \pm 7$ , HS

$34 \pm 10$ ;  $p < 0.05$ ). Among FAB-related MDS subsets, MVD was significantly higher in RAEB-t (MVD  $29 \pm 7$ ; HS  $49 \pm 15$ ) as compared to RA, RARS and RAEB (MVD  $20 \pm 7$ ; HS  $32 \pm 10$ ) ( $p < 0.05$ ). A significant difference in terms of HS was observed between patients with in vitro leukemic growth and patients with non-leukemic growth (HS  $40 \pm 11.2.47$  vs  $28.4 \pm 4.93$ ;  $p < 0.05$ ) and a trend towards significance was seen between MVD/HS and blast count. No correlation was observed between MVD/HS, the international prognostic scoring system (IPSS), and chromosome abnormalities. In summary our data demonstrate that newly formed blood vessels can be found in increased amounts in MDS with respect to normal or reactive BM. This increase is particularly marked in RAEB-t and in MDS with a leukemic pattern of growth in vitro. These observations may suggest a neoangiogenesis role in the pathogenesis and progression in acute leukemia of MDS.

### **C062 CYCLOSPORIN-A MAY IMPROVE IN VITRO CIRCULATING HEMATOPOIETIC PROGENITORS GROWTH AND AFFECTS CD4 CELLS EXPRESSING INTERFERON- $\gamma$ OF HYPOPLASTIC MYELODYSPLASTIC PATIENTS**

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The mechanism of cytopenia in hypoplastic MDS and refractory anemia (RA) is still poorly understood. T-cell clones spontaneously producing inhibitory cytokines have been reported in blood and marrow of MDS patients and may account for increased apoptosis of MDS hematopoietic progenitors. Various immunosuppressive treatments have been sporadically and successfully used in hypoplastic MDS and RA patients. We have investigated the effect of cyclosporin-A (CSA) on circulating hematopoietic progenitors and on CD4 cells expressing interferon- $\gamma$  (IFN- $\gamma$ ) of patients with hypoplastic MDS (n=10) or RA (n=10) *in vitro*. By flow cytometry, circulating CD34<sup>+</sup> cells were found significantly decreased in both hypoplastic MDS (mean value  $\pm$  SEM:  $2930 \pm 1007$ /mL of blood;

$p=0.009$ ) and RA patients ( $3144\pm 1063/\text{mL}$ ;  $p=0.01$ ) compared to normal donors ( $5656\pm 474/\text{mL}$ ). Using methylcellulose colony assay, circulating colony forming-unit cells (CFU-C) were even more decreased in patients with hypoplastic MDS and RA compared to normal donors ( $52\pm 13$  and  $93\pm 24/\text{mL}$  vs  $276\pm 27$ ;  $p<0.001$ ). Circulating  $\text{CD34}^+$  cells and CFU-C of hypoplastic MDS were lower but not significantly different than those of RA patients ( $p=0.1$ ). CSA, at a concentration comparable to that found after *in vivo* administration ( $500\text{ ng/ml}$ ), significantly increased circulating CFU-C colony growth of hypoplastic MDS ( $6.8\pm 1.7$  vs  $13.1\pm 3.2$  without and with CSA, respectively;  $p=0.036$ ) but not of RA patients ( $24.4\pm 7$  vs  $36.9\pm 10$ ;  $p=0.3$ ). Using two color intracellular staining for CD4 and IFN- $\gamma$ , we documented that CSA pretreatment of circulating lymphocytes, activated with phorbol myristate acetate (PMA)-ionomycin, significantly decreased the number of circulating CD4 cells expressing IFN- $\gamma$  in patients with hypoplastic MDS ( $9.6\pm 2.5$  vs  $1.4\pm 0.5$  without and with CSA;  $p=0.001$ ) but not in RA patients ( $5.5\pm 3$  vs  $1.2\pm 0.2$ ;  $p=0.1$ ). We conclude that: 1) the deficiency in the circulating hemopoietic progenitor compartment of hypoplastic MDS patients may be in part lymphocyte-mediated; 2) CSA may improve hematopoietic progenitors growth in hypoplastic MDS by reducing lymphocyte-mediated hematopoietic suppression.

### C063 OCCURENCE OF SECONDARY MDS/AML IN PATIENTS WITH ACUTE PROMYELOCYTIC LEUKEMIA (APL)

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Myelodysplastic Syndromes and Acute Myelogenous Leukemias secondary to chemotherapies (sMDS/sAML) are frequent in patients cured from a previous neoplasia. From 1/89 until 9/98, 87 newly diagnosed APL patients were enrolled in our Institution in 2 consecutive protocols: 28 patients were treated according to GIMEMA 0389 protocol (chemotherapy alone), 59 pts ac-

ording to AIDA protocol (chemotherapy + ATRA). We report 5 cases with APL in molecular Complete Remission (CR) who developed a sMDS followed by sAML in 3 cases. Case 1 (GIMEMA 0389) after 48 months of 1<sup>st</sup> CR showed an evolution in sAML [FAB M4,  $t(10;11)(p14;q21)$ ] with trilineage dysplasia: she achieved a CR with intensive chemotherapy, but died during consolidation from infection. Cases 2 and 3 (both AIDA protocol), after a 1<sup>st</sup> CR of 43 and 46 months respectively developed a s/MDS (RAEB, monosomy 7 in 1 case, karyotype failed in 1 case): both received supportive care only and one is still alive in MDS phase after 10 months, while one evolved in sAML after 2 months and died 1 month later. Case 4 (GIMEMA 0389) had a relapse of APL after 13 months and achieved a 2<sup>nd</sup> molecular CR with ATRA + chemotherapy followed by Autologous Bone Marrow Transplantation (ABMT): after 33 months of 2<sup>nd</sup> CR he developed a sMDS [RAEB,  $\text{del}(5q-)$ ] followed by sAML (FAB M4) after 2 months. He received supportive care only due to heart impairment, and died 5 months later from progressive disease. Case 5 (AIDA protocol) had a 1<sup>st</sup> molecular relapse of APL after 9 months of 1<sup>st</sup> CR and achieved a 2<sup>nd</sup> molecular CR with ATRA alone followed by ABMT. She relapsed as APL after 11 months of 2<sup>nd</sup> CR and achieved a 3<sup>rd</sup> CR with ATRA+chemotherapy: 2 months later, she developed a sMDS (RA, normal karyotype) with peripheral cytopenia and trilineage marrow dysplasia and underwent allogeneic bone marrow transplantation after 5 months of supportive care. All 5 cases were negative for PML/RARa hybrid gene when sMDS/sAML was evidenced. In conclusion, as the occurrence of sMDS/sAML in APL patients with molecular CR is an emerging problem with the improvement of outcome after ATRA, a careful evaluation of marrow dysplasia is warranted in the follow-up of those patients.

## BIOLOGY

### **C064 STI571 ERADICATES BCR/ABL+ LEUKEMIC CELLS IN NUDE AND SCID MICE, DEPENDING ON THE INITIAL TUMOR LOAD**

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STI571 is a selective inhibitor of both the ABL kinase as well as of the BCR/ABL oncogenic kinase and has been shown to inhibit the proliferation of BCR/ABL+ cell lines as well as of fresh cells obtained from CML patients. We recently showed that STI571, when administered p.o. at 160 mg/kg three times per day for 11 days, causes continuous in vivo block of the BCR/ABL kinase activity and eradicates the growth of  $50 \times 10^6$  human BCR/ABL+ leukemic cells (KU812) injected in the flank of nude mice. When the treatment was initiated 24 hours after leukemic cell injection, eradication of tumor growth was obtained in all treated animals (18/18). When animals were treated 8 days after injection, with an estimated tumor load of  $3-400 \times 10^6$  cells, nodules disappeared in all animals within 10 days; however in 41% of treated animals (9/22) the tumor reappeared between 16 and 19 days after treatment discontinuation. This difference is statistically significant ( $p = 0.0018$ ). Treatment at day 15 (tumor load of  $10^9$  cells) resulted in regression of nodules but no animal was cured. Retreatment of relapsed animals did not obtain permanent eradication of leukemic growth, and in vivo STI571-mediated inhibition of the BCR/ABL kinase activity was reduced or absent. Prolongation of treatment duration from 11 days to 18 days also failed to decrease the risk of relapse. BCR/ABL+ leukemic cells obtained from relapsed animals were retested in vitro for sensitivity to STI571 by 3HTdR uptake and showed in general the same sensitivity as parental cells. The main variable able to predict the efficacy of STI571 in eradicating leukemic growth in

this nude mouse model is represented by the initial tumor load; leukemic cells obtained from relapsed mice do not appear intrinsically resistant to STI571. These data show that the number of leukemic cells present at the beginning of treatment represent an important variable, even when considering a specific anti-leukemic treatment. Additional data obtained in SCID mice injected with fresh CML cells from patients in chronic phase support the above mentioned results, and will also be presented.

### **C065 PULSE OF LOW DOSE FLUDARABINE AVOIDS SELECTION OF DOXORUBICIN RESISTANT CLONE IN K562 CELL LINE**

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Fludarabine (F-ARA-A) is a purine analogue with a cell cycle specific activity. It inhibits DNA synthesis by two different mechanisms: 1) direct blocking DNA elongation and 2) decreasing cellular dNTPs through inhibition of ribonucleotide reductase. There are also some evidences that Fludarabine could be incorporated into mRNA inhibiting gene expression. It is well known that K562 cell line have a baseline expression of gp170 and hence it is possible to select doxorubicin resistant clone after exposure to increasing level of the drug. Gp170 is the best studied mechanism supporting Multi Drug resistance (MDR). It is a membrane efflux pump that impairs accumulation of widely-used lipophilic drugs including anthracyclines, epipodophyllotoxins and vinca alkaloids. In the past Verapamil and its analogues were frequently used in vitro to revert gp 170 action but in vivo they showed troublesome side effects. In our laboratories parental K562 cells were exposed to Doxorubicin (DXR) at concentration of 25 nM combined to weekly pulse of 0,5 µg/ml (1,38 µM) of Fludarabine. In this case no selection of MDR clones occurred. On the contrary, the treatment of K562 parental cells by DXR alone allowed the emergency of drug resistant clones. At the concentration used, Fludarabine did not modify the doubling time and growth pattern of K562 cells but increased the

percentage of S phase up to 80%. Cytofluorimetric analysis, performed using MRK16 antibody showed that pulse of Fludarabine treatment revert the K562 gp170 baseline expression. Moreover immunocytochemical analysis, performed using JSB1 antibody on both, DXR and DXR plus Fludarabine treated cells, confirmed that inhibition of selection was due to the block of gp 170 expression by Fludarabine. In fact, while the resistant cells obtained after direct exposition to DXR were markedly positive to JSB1 antibody, those treated with DXR plus Fludarabine were not. If *in vitro* results will be further confirmed, low doses of Fludarabine can result in a useful clinical approach.

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## **C066 CHARACTERIZATION OF THE BIOLOGICAL EFFECTS OF A NOVEL RETINOID ON AML CELLS**

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Retinoids are modulators of cellular proliferation and differentiation in many cell types and their diverse effects are mediated by three distinct isoforms of receptors RAR (α, β, γ). In this study we characterized a novel derivative of retinoic acid (pat. WO 97/02030) and compared its activity to that of known retinoids. The new molecule and known retinoids have similar chemical properties, by studies with HPLC and spectrophotometer analysis, but the new compound does not own the typical sensitivity to the light. Acute promyelocytic leukemia (APL) cell lines, HL60, NB4 and KASUMI, as well as primary (APL) cells were cultured in RPMI 1640 with 15% FCS for 3, 4, 6 days, supplemented with the retinoic acid new derivative 10mM, 100 nM, 1 mM or *all-trans* retinoic acid (ATRA) at the same doses. After that time, we evaluated: cells counts, morphology, flow cytometric analysis of cell-cycle, detection of apoptosis evaluating

Annexin V binding. Our results showed that the new molecule blocked proliferation at the same extent as ATRA 1 μM and 100 nM, while it resulted toxic at 10 μM. After 4 days of culture, cells in S-phase were only 11.72% when exposed to the new retinoid 1 μM, compared with 42.49% of control cultures. Consistent data were obtained with ATRA 1 μM. Total cell number was decreased after treatment with novel retinoid. Annexin V test demonstrated induction of cell line apoptosis: 30% of apoptosis compared with 9.96% of control in HL 60, 23% vs 6% in Kasumi, the percentage was not significant in NB4 cells; as well as in primary APL cultures. These data were supported by the morphological observation of apoptotic bodies as well as by the appearance of pre-G1 peak in flow cytometric analysis of cell-cycle. Moreover, we performed transient transfection in COS-1 cells with the expression vector pSG5/RARα and PSG5/RXR, showing that the new molecule interacted with the nuclear retinoid receptors RXR, and the effect amplified by interaction with heterodimer RXR-RARα. Preliminary studies with fractionation of HL 60 and NB4 nuclear extracts over FPLC showed that the new molecule binding affinity for the endogenous nuclear receptors is stronger than that of ATRA. These observations helped to analyze whether the new retinoid implies different therapeutic strategies, so to be possibly used in alternative to the known retinoids in the treatment of resistant (APL).

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## **C067 IDENTIFICATION AND ISOLATION OF A NORMAL BIPOTENT ERYTHROID- MEGAKARYOCYTIC CELL PRECURSORS**

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Based on several *in vitro* data supporting the existence of a bipotent, erythroid and megakaryocytic, cell precursor, we sought to identify such a cell in normal tissues.. First, to clarify until when hematopoietic cells remain bipotent for erythroid and megakaryocytic differentiation, we analyzed (by RT-PCR) the expression of erythroid (α-

and  $\beta$ -globin and erythropoietin receptor, *EpoR*)- and megakaryocytic (acetylcholine esterase, *AchE*, glycoprotein IIb, *GpIIb* and thrombopoietin receptor, *Mpl*) genes in single colonies derived from early (BFU-E, CFU-Mk and CFU-GM) and late (CFU-E) murine progenitor cells. Almost all (90 %) the erythroid bursts and megakaryocytic colonies (out of a total of 70 colonies) and none of the CFU-E- and CFU-GM-derived colonies (out of 30-40 colonies) investigated expressed both erythroid and megakaryocytic genes. These data suggested that the bipotent cell precursor is intermediate between BFU-E and CFU-E. Then we analyzed by FACS the expression of TER-119 and 4A5 (two surface markers specific for erythroid and megakaryocytic cells) in marrow and spleen cell suspensions from normal mice and from mice recovering from the anemia induced by phenyl-hydrazine (PHZ). TER-119<sup>+</sup>/4A5<sup>+</sup> double positive cells were identified in the marrow from normal mice (1.3±0.6%) and in the marrow (3.8±0.8%,  $p < 0.01$ ) and spleen (1.3-8.3 %) from PHZ-treated animals. TER-119<sup>+</sup>/4A5<sup>+</sup> cells (30 or 65 % pure by FACS re-analysis), isolated by chemical (18 hrs in the absence of growth factors) or physical (immunomagneting selection or cell sorting) means from the spleens of PHZ-treated mice, contained benzidine-negative blasts expressing low levels of  $\beta$ -globin and *GpIIb* by RT-PCR. The purified cells generated single Ter-119<sup>+</sup> and 4A5<sup>+</sup> cells with a clear erythroid and megakaryocytic morphology when cultured for 24-48 hrs with EPO or TPO. In conclusion, we have identified *in vivo* a TER-119<sup>+</sup>/4A5<sup>+</sup> cell population which meets the criteria for the bipotent cell precursor and may play an important role in the recovery from the anemia induced by PHZ.

## C068

### CD40 LIGATION ON MALIGNANT PRECURSOR B CELLS CAN INDUCE A POTENT CHEMOATTRACTANT ACTIVITY FOR ANTI-LEUKEMIA CYTOTOXIC T CELLS

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B-cell acute lymphoblastic leukemia (ALL) cells are ineffective antigen-presenting cells (APC) and fail to induce significant immune responses. By crosslinking CD40 expressed by the leukemia cells using the soluble form of the ligand (CD40L), we have been able to improve their immunogenicity and to develop a methodology for the generation and expansion of autologous anti-leukemia specific cytotoxic T cells (CTL). These results prompted us to envision the use of CD40-stimulated leukemia cells (CD40-ALL) as tumor cell vaccines. Since a critical requirement for a successful tumor cell vaccination is the attraction of anti-tumor effector T cells to the sites of disease, we thus sought to determine whether CD40-ALL cells secrete T cell-chemo-attractants, in particular, for antigen-specific memory T cells. An extensive study of chemokine expression in ALL cells showed that, in all patients tested (n=24), the chemokine MDC (macrophage-derived chemokine) was specifically induced by CD40 crosslinking. This induction was observed both at mRNA and protein level, with secreted MDC ranging from 13.9 to 18.8 ng/ml. As MDC has been described as a very efficient chemoattractant for activated T cells, we examined the anti-leukemia specific T cells generated in our system for the reactivity to this chemokine and in particular for the expression of CCR4, which has been described to be the specific receptor for MDC. Anti-leukemia T cells, which are mostly CD8<sup>+</sup> T cells, do express CCR4 (mRNA and protein) and respond to MDC by calcium mobilization and migration through endothelium. This reactivity was observed in response to both recombinant MDC and supernatant from CD40-ALL cultures. In conclusion, we dem-

onstrate that a single physiologic signal (i.e. CD40 crosslinking) does not only improve the immunogenicity of ALL cells but also induce them to produce a potent chemo-attractant for activated T cells. Moreover, ex-vivo generated autologous anti-leukemia CTLs express CCR4 and respond to MDC by migrating through endothelium, underscoring the rationale for the use of CD40-stimulated leukemia cells in vaccination strategies for the treatment of ALL.

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**C069**  
**CELLULAR LOCALIZATION OF HUMAN HERPESVIRUS-8 (HHV-8) AND EXPRESSION OF HHV-8 CELL-HOMOLOGOUS GENES IN HIV NEGATIVE LYMPHOPROLIFERATIVE DISEASES**

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The occurrence of HHV-8 infection in lymphoid tissues is rare and the cell types infected are largely unknown. Recently, HHV-8 genome has been shown to contain genes encoding homologues to cytokines, oncoproteins and cell cycle regulatory and signaling proteins, that have been acquired from the host cell. We explored whether transcription of HHV-8 genes may occur in lymphoid tissues, in vivo, and also studied the cellular localization of the virus by in situ polymerase chain reaction (PCR). Thus, we used reverse transcriptase PCR to look for the expression of the HHV-8 genes homologous to human interleukin-6 (IL-6), cyclin-D, BCL-2 and interleukin-8 receptor (G-protein-coupled receptor-GCR-) in two cases of benign lymphadenopathy with giant germinal center hyperplasia and increased vascularity and in two cases of Castleman's disease (CD). None of these genes was expressed in the case of benign localized CD of hyalin-vascular (HV) type, and only vIL-6 and vCyclin-D were transcribed in the two cases of benign lymphadenopathies. In contrast all four genes were transcribed in the case of multicentric Castleman's disease of plasma cell type (PC) type with aggressive clinical course and in two cases of primary effusion lymphomas (PEL). HHV-8 was localized in lymphoid and

monocyte-macrophage cells scattered in the interfollicular regions of both lymphadenopathies with giant germinal center hyperplasia and increased vascularity, but not in endothelial cells. Our study reports, for the first time, that HHV-8 genes homologous to cell genes, may be transcribed in lymphoid tissues in vivo, out of the KS and AIDS settings. The differential expression of HHV-8 genes homologous to cellular genes involved in cell proliferation (v-cyclin-D and vGCR) and apoptosis (vIL-6 and vBCL-2) suggests that they may influence the lymphoproliferative process associated with this herpesviral infection. The distribution of HHV-8 infected cells in non neoplastic lymph nodes outside of the germinal centers resembles that of Epstein-Barr virus-infected cells in the lymph nodes in the course of infectious mononucleosis.

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**C070**  
**THE CARBOXYTERMINAL REGION OF G-CSF RECEPTOR TRANSDUCES PHAGOCYTOSIS SIGNALS**

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Granulocyte colony stimulating factor (G-CSF) induces proliferation and maturation of myeloid progenitor cells, but plays also an essential role in promoting functional activities of mature granulocytes, like phagocytosis. We have demonstrated that Shc, Syk and Lyn kinases are independently tyrosine phosphorylated after G-CSF stimulation of 32d murine myeloid cells transfected with human wild type (WT) G-CSF-R. We showed that tyrosines 764 and 729 of G-CSF-R cytoplasmic domain are crucial for activation of Shc and Syk, respectively. Syk kinase role in granulocytic precursor proliferation and maturation has not been completely clarified. It has been demonstrated that Syk kinase is required for Fcγ receptor mediated phagocytosis in monocytes/macrophages and that in a COS-1 cell model system, co-transfection of Syk kinase with FcγRI and its g chain subunit enhances phagocytosis 5-7 fold, while co-transfection of Syk with FcγRIIA enhances phagocytosis ~2 fold. In order to examine

the influence on granulocytic function of G-CSF induced Syk activation, we analyzed the pattern of tyrosine phosphorylation of 32d myeloid cells WT/G-CSF-R transfectants, and DA/G-CSF-R natural occurring truncated mutant (D715). In the latter mutant the ITAM (immunoreceptor tyrosine-based activation motif)-like motif of G-CSF-R is lacking. The ITAM motif contains the binding sites for Syk. We then stimulated G-CSF-R transfectants and mutants with G-CSF 100 ng/ml for 10 minutes and analysed at light microscopy phagocytosis of sheep red blood cells, after 30 minute incubation in a 37 °C shaking water bath. DA/G-CSF-R mutant did not show Syk phosphorylation after stimulation. 32d WT/G-CSF-R transfectants had a baseline phagocytosis of  $10 \pm 2\%$  (300 cells scored), but after G-CSF stimulation  $44 \pm 5\%$  of cells were phagocytic. The truncated DA mutant had only 1% of cells showing phagocytosis, and no increase was obtained after stimulation with G-CSF. A possible interpretation model proposes Src tyrosine kinase interaction with Syk kinase as an early event in signaling cascades responsible for transmitting extracellular signals from surface receptors to cytoplasmic pathways crucial for functional response. In our knowledge, this is the first evidence indicating G-CSF-R signals phagocytosis through a specific region. As we demonstrated that the same region of the receptor is responsible for Syk activation, we concluded that the phagocytic activity stimulated by G-CSF in 32d WT/G-CSF-R transfectants is modulated by the carboxy-terminal region of the receptor via Syk.

## C071

### DIAMOND-BLACKFAN ANEMIA: ROLE OF THE RPS19 GENE IN THE ITALIAN POPULATION

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Diamond-Blackfan anemia (DBA, MIM 105650) is a congenital red cell aplasia,

associated with a wide range of physical abnormalities. Recently, mutations in ribosomal proteins 19S (RPS19) were identified in some DBA patients. The RPS19 gene, located within 19q13.2 region, encodes ribosomal protein S19, the first structural ribosomal protein found to be involved in a human disease. RPS19 includes six exons, the first of which untranslated. Mutations were found in 25% of DBA patients. To evaluate the impact of mutations in RPS19 in pathogenesis of DBA in the Italian population, we screened for mutations 49 Italian patients; mutations were identified in 9 families (18.4%), always in heterozygosity (deletion of the entire gene, -633insAGCC, 53insAGA, 237insG, W52X, R62W, R94X, R101H, IVS5 1G→A). Most mutations were located in exon 4 (5/9), two are shared by more than one patient (not consanguineous individuals) and/or by patients from other population. Two cases were familial; the others were sporadic. One parent shows isolated macrocytosis, suggesting variable expressivity of DBA. High ADA levels were found in most patients who carry a mutation in RPS19, but also in patients with a normal gene. Similarly, we have not found mutation-phenotype correlations. It is likely that other factors contribute to the variable expressivity of DBA, as shown by two non-concordant monozygotic twins from our panel. The identification of DBA gene has immediate implications for clinical practice: it allows molecular diagnosis of the disease and genetic counselling in DBA families: parents with a sporadic DBA child can be reassured on the risk of recurrence, whereas prenatal diagnosis is possible for inherited mutations.

## C072

### BONE MARROW ERYTHROID EXPANSION IN $\beta$ THALASSEMIA. EVALUATION BY FLOW CYTOMETRY

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Studies at the bone marrow level represent a crucial aspect in the understanding of many hematological abnormalities included the  $\beta$  thalassemsias. Beta thalassemsia major is an hereditary disease characterized

by ineffective erythropoiesis which leads to expansion of the erythron and severe anemia due to intramedullary programmed cell death (apoptosis) of the erythroid precursors. In this study we have defined quantitatively by flow cytometry, the erythroid expansion in 78 transfusion-dependent  $\beta$  thalassemic patients evaluating the whole bone marrow mononuclear cells reactivity (absolute number) to the CD36 and the CD71 antigens. The ineffective erythropoiesis has been also quantified (absolute number) evaluating the early apoptotic (Annexin V positive) erythroid precursors (CD45 negative). Our results show that the extent of the erythroid hyperplasia directly correlates with the extent of the early apoptosis (CD36 vs CD45-AnV+  $r = 0.90$   $p < 0.0000$ ; CD71 vs CD45-AnV+  $r = 0.90$   $p < 0.0000$ ). We were also able to define three increasing levels (Low, Medium, High) of erythroid expansion. Statistically significant differences were observed between the different erythroid hyperplasia levels when the reactivity to different antigens (CD36, CD71, CD45-/AnV+, CD16, CD34) and several clinical features (age, n° of transfusions, liver iron concentration, myeloid mass/ erythroid mass, chelation irregular, splenectomy, presence of acute chronic hepatitis) were considered.

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## **HODGKIN AND NON-HODGKIN LYMPHOMAS**

### **C073 TUMOR BURDEN (TB) AS PRIMARY PROGNOSTIC FACTOR IN HODGKIN'S DISEASE (HD)?**

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The volume of TB present at diagnosis was measured in 121 patients with HD, treated in the last 10 years (median follow-up: 44 months). Patients were selected only through the availability of both magnetic records of total body CT scan and ultrasonographic dimensional evaluation of the superficial lymph node not included in the CT scans. CT scans had to be serial and contiguous and the identified lesions had to be tridimensionally reevaluated and measured through the elaboration resources of the CT equipment. In the majority of patients the enlarged lymph nodes not included in the CT images were measured with a Toshiba ultrasound instrument by means of a 7 MHz probe. The TB of the 121 patients, resulting from the sum of the volumes of all the lesions measured on the CT and US scans, ranged from 4 and 985 ccm ( $175.114 \pm 195.055$ ). The prognostic value of TB was investigated in relation to time to treatment failure (TTF). In two distinct multivariate analyses, the first including, the second not including serum  $\beta$ 2-microglobulin ( $\beta$ 2-m) among covariates (because of its lack in many clinical records at diagnosis), the parameters statistically most related with prognosis were the following (statistical P between brackets): TB (0.0006),  $\beta$ 2-m (0.008), ESR (0.012), Karnofsky index (0.019); or, without  $\beta$ 2-m, TB (<0.0001), ESR (0.0003) and serum fibrinogen (0.025). TB showed to be well-correlated with the majority of the biologi-

cal and clinical parameters currently used in the initial evaluation of the disease, especially with bulky disease, number of involved lymph node regions, LDH, ESR, hemoglobin, Karnofsky index. However, its predictability from these variables is rather low ( $R^2 = 0.534 \div 0.667$ ). Therefore TB seems to be largely independent of the clinical factors currently considered for clinical uses. This is further confirmed by the direct comparison of the prognostic value of TB and International Prognostic Factor Project for Hodgkin's Disease (Hasenclever D et al., JCO 1998, 339, 1506). At the bivariate analysis vs. TTF TB was the only statistically significant factor ( $P < 0.0001$ ) while the IPFP score showed a P of 0.6207. TB is emerging as a new prognostic factor in HD, more powerful than and largely independent of those well-known and used so far.

### **C074 TREATMENT OF EARLY-STAGE HODGKIN'S DISEASE WITH ABVD AND LIMITED RADIOTHERAPY: ANALYSIS OF EFFICACY AND LONG-TERM TOXICITY**

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The use of combined modality therapy in early-stage Hodgkin's disease can spare staging laparotomy, allows smaller field and lower dose of radiation and reduces the risk of relapse compared to radiation alone. This paper reports on the results and long-term events of a combined modality approach consisting of a brief chemotherapy (CT) and limited radiotherapy (RT) without staging laparotomy in patients with early-stage Hodgkin's disease. This prospective study included 78 patients (20 with clinical stage I and 58 with stage II); 60% of total had mediastinal enlargement, 12% bulky disease, 6% B symptoms and 5% subdiaphragmatic disease. Median age was 33 years (range: 15-64) and median follow-up 56 months. The treatment program consisted of four cycles of the ABVD regimen, as originally described, followed by limited RT on involved sites (44 patients) or involved and contiguous sites of disease (34 patients); radiation dose ranged from 30

to 36 Gy to uninvolved and involved sites, respectively; bulky disease received up to 44 Gy. Gonadal function in women was assessed by hormonal tests and menses evaluation; fertile women were given an estrogen-progesterone combination for ovarian protection, while most of young men had their semen cryopreserved. The treatment program was completed in a median of 6.2 months (range: 5-10). Complete remission (CR) rate was 88% after 4 ABVD (69 patients) and 98% after the adjunctive RT. The 5-year relapse-free survival (RFS) is 97%; two of 3 relapsing patients had achieved only partial remission after ABVD. The 5-year overall survival is 98%; two patients died to date, one of disease progression and one of small cell lung carcinoma. Therapy-related long-term toxic events included two cases of pulmonary fibrosis with symptomatic interstitial disease, one case of dilated cardiomyopathy with cardiac failure (all patients had received mediastinal irradiation), and hypothyroidism requiring replacement therapy in five cases. Fertility was preserved in young women and four normal pregnancies were registered. No cases of secondary leukemia occurred. In early-stage Hodgkin's disease not undergoing staging laparotomy, the combination of a brief CT consisting of 4 cycles of ABVD and limited irradiation was effective and produced a 97% RFS at 5 years. A prolonged monitoring of potential long-term *sequelae* of therapy and evaluation of their impact on the quality of life are mandatory in this curable setting of patients.

**C075**  
**HIGH-DOSE SEQUENTIAL (HDS)**  
**REGIMEN AS SALVAGE TREATMENT**  
**FOR REFRACTORY/RELAPSED**  
**HODGKIN'S DISEASE: A MULTICENTRE**  
**EXPERIENCE**

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The Italian Lymphoma Intergroup (I.L.I.) comprehends most Italian Centres involved in the management of lymphoma. In 1996

I.L.I. Centres started a prospective program with the high-dose sequential (HDS) chemotherapy regimen as salvage treatment for relapsed or refractory Hodgkin's Disease (HD); besides, data from previous experiences gained with HDS in the management of progressing HD were collected. Aims of this study were: i. to evaluate feasibility and efficacy of HDS in relapsed/refractory HD in a multicentre setting; ii. to define overall curability of HD patients by combining cure rate after first line therapy along with salvage with intensive chemotherapy. So far, 46 patients have been enrolled in HDS programs at 8 Italian institutions. The HDS schedule includes the sequential administration of high-dose (hd) cyclophosphamide (7 gr/sqm) followed by peripheral blood progenitor cell (PBPC) harvest, hd-methotrexate (8 gr/sqm), hd-etoposide (2 gr/sqm) and finally hd-mitoxantrone (60 mg/sqm) + L-Pam (180 mg/sqm) followed by PBPC autograft. Main patient characteristics at HDS start were as follows: median age 28 yrs. (range 16-50), stage III-IV= 25 pts., B symptoms=24 pts., BM involvement=6 pts., PS <sup>3</sup>2=9 pts., refractory disease=17 pts., 1<sup>st</sup> relapse=21 pts., >1<sup>st</sup> relapse=8pts. Thirty-seven pts. have completed HDS and are evaluable. There were 3 treatment-related deaths (TRM=8%); 5 more pts. had disease progression; CR was achieved by 5 out of 13 refractory pts, 10 out of 16 pts. in 1<sup>st</sup> relapse and 6 out 8 pts. >1<sup>st</sup> relapse. Overall, CR was achieved by 21 out of 37 evaluable pts. (57%); so far, there was no relapse among pts. in CR. In conclusion, this preliminary analysis shows that HDS is feasible in a multicentre setting; its toxicity can be considered moderate, since all patients were variably pretreated; the CR rates obtained both in relapsed and refractory pts. are promising in terms of therapeutic efficacy of the scheme.

**C076**  
**10-YR FOLLOW UP IN 246 PATIENTS WITH ADVANCED STAGE DIFFUSE LARGE CELL LYMPHOMA (DLCL) TREATED WITH STANDARD 12 WEEK CHEMOTHERAPY: LATE RELAPSES AND LATE TOXICITIES**

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**Introduction:** new intensive approaches give promising results in the treatment of DLCL, but they need to be compared with the long term results achievable with prior regimens. **Patients and Methods:** in order to define the cure rate, late toxicities and late relapses, a long term analysis was performed on 246 patients (age 15-60) with advanced stage DLCL treated with MACOP-B (200) or VACOP-B (46) regimens from 1986 through December 1993. **Results:** median follow up was 115 months. Seventy-six % achieved a CR, 9% a PR, 11% showed a NR and 4% died of toxicity. 10-yrs OS is 58% and 10-yrs FFS is 53%. Among 185 CRs 52 pts relapsed. Late relapses (>2 yrs from the completion of therapy) occurred in 9 pts. Seven pts relapsed with aggressive DLCL, one with follicular lymphoma and one showed a Hodgkin's disease. Five are alive in second CR and four died of lymphoma. Late toxicities (>1 year from the completion of therapy) were recorded in 28 pts: 7 femoral head osteonecrosis, with a median time off chemotherapy of 15 months; 6 cardiac toxicities (4 cardiomyopathies, one ischemic disease and one arrhythmia) at a median time off therapy of 60 months; 5 severe peripheral neuropathy, one renal failure, one pulmonary fibrosis and one viral encephalitis. Seven pts, in CR of lymphoma, showed a second cancer, with a median time off therapy of 70 months: 2 developed acute myelogenous leukemia and died of this disease, 5 had solid tumors (2 lung, 1 breast, 1 gastric and one head and neck cancer) and two of them subsequently died of that. Actuarial risk to develop a second cancer was 6% at 10 years. **Conclusions:** 50% of

pts with advanced stage DLCL are cured with conventional chemotherapy. However late relapses may occur even after 8 years. 11% of pts developed late toxicities. Secondary neoplasia risk is not negligible. Long term follow up data must be reported to draw definite conclusions in the outcome of DLCL patients and may be useful as historical comparison in new trials.

**C077**  
**RISK-FACTORS T IN DIFFUSE LARGE CELL LYMPHOMA AT FIRST RELAPSE. A STUDY OF THE ITALIAN INTERGROUP FOR LYMPHOMAS**

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**Introduction:** The objective of the present study was to identify risk groups in adult patients (pts) with diffuse large cell lymphoma (DLCL) at first relapse (R1). **Methods:** we studied 448 pts representing >90% of relapses observed in a decade (88-97) of prospective trials of initial chemotherapy by 4 multicenter groups and by 3 centers in Italy. Median time to R1 was 381 days from diagnosis (Dx), 92% relapsed <3.5 yrs from Dx and 96% were initially treated >3.5 yrs ago. Median age at R1 was 55 yrs (16-85) and median follow-up is 3.3 yrs. Overall response (CR+PR) was 63% (similar in various salvage regimens) and high-doses + stem cell transplant (HDSCT) was added in 89 pts. At 3 yrs, overall survival (OS) was 35% and progression-free survival (PFS) was 26%. OS and PFS were compared (log-rank) by: histology (WF, high vs intermediate; REAL, large B vs peripheral-T vs anaplastic CD30+), phenotype (B vs T), time to R1 (<1 yr vs >1yr from Dx), age at R1 (<65 vs >65), LDH at R1 (N vs >N), stage at R1 (I-II vs III-IV), performance status (PS) at R1 (0-1 vs >1). Age at R1, overall response to salvage chemotherapy and HDSCT intensification were included in the Cox models to adjust factors related to OS and PFS at univariate analysis. **Results:** Univariate:

OS and PFS were related with WF histology, with time to R1 and with age-adjusted IPI factors at R1 (LDH, stage and PS). Multivariate: <1 yr to R1 (RR=1.7, CI 95%=1.3-2.2) and age-adjusted IPI >1 at R1 (RR=2.4, CI 95%=1.8-3.2) were adverse factors (AFs). **No. of AFs** at R1 (**0,1,2**) identified 3 risk groups (p<0.0001). We report the actuarial % **OS** and **PFS** at 3 yrs in each risk group for 399 pts (**Overall**), for 252 achieving CR or PR (**Sensitive Relapses**) and for 83 pts who were intensified by HDSCT (**Intensified**).

No.AFs	Overall			Sensitive Rel.			Intensified		
	No.	OS	PFS	No.	OS	PFS	No.	OS	PFS
<b>0</b>	122	54	40	92	76	50	26	63	50
<b>1</b>	173	29	26	120	51	37	44	59	44
<b>2</b>	104	6	5	40	13	13	13	15	8

**Conclusion:** in adults with DLCL at first relapse time to relapse and age-adjusted IPI should be balanced in comparative studies of salvage therapy since they predict risk independently from age, response to treatment and intensification with HDSCT.

## C078

### THE ROLE OF POSITRON EMISSION TOMOGRAPHY (PET) IN THE MANAGEMENT OF LYMPHOMA PATIENTS

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Treatment of both Hodgkin's disease (HD) and aggressive non-Hodgkin's lymphoma (NHL) with abdominal presentation at the time of diagnosis is often followed by detection of residual masses by computed tomography (CT). However, CT is generally unable to discriminate between residual tumor and fibrosis/necrosis. We investigated the ability of fluorine-18 fluorodeoxyglucose positron emission tomography (PET) to differentiate residual active tumor tissue and fibrosis. Forty-four patients with HD or aggressive NHL presenting abdominal involvement (41% with bulky mass) were studied with CT and PET at the end of chemotherapy ± radiation therapy. After treatment, 7 patients had negative PET and CT, none of whom relapsed. The remaining 37 patients

all had positive CT (abnormalities £ 10%). The 13 who also had positive PET all relapsed (100%). By contrast, there was only 1 (4%) relapse among the 24 patients who were positive at CT but negative at PET. The 2-year actuarial relapse-free survival rate was 95% for those with negative PET compared with 0% for positive PET patients (p < 0.000000). In lymphoma patients with abdominal masses who present CT positivity at restaging, PET should be considered the noninvasive imaging modality of choice for differentiating recurrence or residual disease from fibrosis.

## C079

### A PILOT-STUDY ON A SEQUENTIAL VARIANT OF PROMECE-CYTABOM CHEMOTHERAPY WITH IDENTICAL DOSE INTENSITY AND THREEFOLD DOSE SIZE

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The results of high-dose chemotherapy followed by rescue with bone marrow progenitor cells are generally ascribed to the high dose size (DS) of the drugs given. However, a contemporary marked increase in the dose intensity (DI) is always involved. To the aim of a future comparison of the role of DS and DI in non-Hodgkin lymphomas a variant of Fisher's ProMECE-CytaBOM regimen was designed, in which the projected cumulative drug DI's are the same as in the original schedule but the DS's are three times higher. Doses in mg/sm and days of administration are the following: CTX 1950 iv 1°, 64°; MTX 360 iv 15°, 78°; VCR 1.4 iv 15°, 78°, 43°, 106°; VP-16 360 iv 29°, 92°; EPI 120 iv 29°, 92°; BLM 15 iv 43°, 106°; ARA-C 900 iv 50°, 113°. This regimen was administered to 29 untreated and 7 relapsed patients with non-Hodgkin's lymphomas presenting intermediate- and high-grade histology according to the Working Formulation. Clinical stage was I in 1

patient, II in 7, III in 5 and IV in 23; 10 presented B symptoms; the IPI score was 0-2 in 29 cases and  $\geq 3$  in the remaining 7. Of the 29 untreated patients 16 achieved CR, 8 PR, 4 PD and 1 was early withdrawn from the study due to acute viral hepatitis; subsequently, 4 relapsed and 3 died (2 of disease progression, 1 of causes unrelated with the disease). In the pretreated group 3 patients obtained CR, 2 PR and 1 PD; 3 of these died (1 of progressive disease, 1 of a new relapse, 1 of myocardial infarction during therapy). With a 16-month median follow-up, OS was 0.82 and DFS 0.79. G-CSF was administered to all the patients but 2 with a median delivery during the whole regimen of 8400  $\mu\text{g}$  per patient. Mean cumulative DI was 0.81. Grade 3-4 hematological toxicity consisted in 3 cases of anemia, 8 of leucopenia and 2 of thrombocytopenia; the same grade nonhematological one involved liver in 2 cases, heart in 1 (the above mentioned death), digestive mucosa in 2 and peripheral nerves in 1. In conclusion, the iso-DI sequential variant of the ProMECE-CytaBOM regimen can be considered feasible, relatively not much toxic, and can be administered on an outpatient basis. A limited use of G-CSF is required (about 3 vials after each drug administration). A randomized trial with the original ProMACE-CytaBOM regimen can be proposed.

## CO80

### AUTOLOGOUS STEM CELL TRANSPLANTATION (ASCT) FOR ADULT LYMPHOBLASTIC LYMPHOMA (LBL) IN FIRST REMISSION: RESULTS FROM A RANDOMIZED TRIAL OF THE EUROPEAN GROUP FOR BLOOD AND MARROW TRANSPLANTATION (EBMT) AND THE UK LYMPHOMA GROUP (UKLG)

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The aim of this multi-center study was to compare the effectiveness of conventional treatment with high-dose therapy (HDT) plus ASCT in improving survival and DFS of 1<sup>st</sup> remission adult LBL patients. From November 1992 to April 1997, 119 adult pts with LBL from 33 European centres en-

tered the EBMT/UKLG LY01 study. All pts were treated with standard remission/induction therapy. Responding pts were randomised to either conventional dose consolidation/ /maintenance therapy (CT) or HDT and ASCT. In some centres, pts with HLA-identical sibling donors were registered for the trial, but proceeded to allogeneic BMT without randomisation. Randomised pts relapsing after CT who responded to salvage therapy received ASCT in 2<sup>nd</sup> remission. Pts characteristics: Male 83, female 36; median age 26 years (range 14 to 65); Ann Arbor stage III/IV 83, T cell 80; LDH elevation 64. Results: Response to induction therapy: CR 67 (56%), PR 31, NR/PD 9; toxic death 1; protocol violation 1; inevaluable 7. Sixty-five pts were randomised (31 ASCT, 34 CT). Reasons for failure to randomise: NR/PD 16; toxicity of induction therapy 5; allogeneic BMT 12; protocol violation 6; patient refusal 12. For randomised pts, the 3yr actuarial relapse free survival is 56% in the ASCT arm, versus 14% in the CT arm ( $p=0.08$ ). Corresponding 3yr actuarial overall survivals are 62% and 52% respectively ( $p=0.98$ ). Conclusions: These results suggest that ASCT is superior to conventional dose chemotherapy as post-remission therapy for adult LBL. The fact that overall survival is the same for both arms may reflect the effectiveness of ASCT in 2<sup>nd</sup> remission for patients who relapse after conventional consolidation therapy.

## CO81

### CIS-PLATINUM, IDARUBICIN, PREDNISONE (CIP) AS CONSOLIDATION THERAPY FOR ELDERLY NHL PATIENTS AFTER P-VABEC REGIMEN. AN ITALIAN MULTICENTER RANDOMIZED STUDY

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**Background:** In an our previous phase II study the P-VABEC regimen resulted an active and well tolerated therapy for elderly patients (pts) with diffuse large cell NHL

(JCO 11- 2363,1993). In spite of a high rate of CR the overall response rapidly decreased for a remarkable incidence of relapses.

**Purpose:** To evaluate the activity and toxicity of CIP consolidation therapy after P-VABEC versus standard P-VABEC regimen in a prospective a randomized study.

**Patients and methods:** From October 1995 to April 1999 were enrolled 152 previously untreated pts with diffuse large cell NHL (according to REAL), median age of 70 yrs (range 60-85), stage II-IV. The CIP schedula, started 21 days after the end of P-VABEC, consisted of : Cis-platinum (40mg/td day 1), Idarubicin (15mg/m<sup>2</sup> day 8), and Prednisone (40mg/td days 1-4/ 8-11) repeat every 21 days for a total of 3 cycles. So far 113 patients are evaluable for the response, 65 pts randomized for P-VABEC (group 1) and 48 for P-VABEC-CIP (group 2). According to the age-adjusted International Prognostic Index (IPI) 52 pts were considered as Low Risk (IPI 0-1) and 61 as High Risk (IPI 2-3). **Results:** With a median follow up of 18 months (range 1-46) the CR rate, OS and EFS at 2 years were 63%, 58%, 54% in the group 1 and 62%, 72%, 68% in the group 2 (p= ns). If we consider pts with High Risk the OS was 42% and 80% (p=0.08) and the EFS was 40% and 57% (p=0.27) respectively in the group 1 and group 2. In the Low Risk group the OS was 95% and 73% (p=0.10) and EFS was 78% and 76% (p=0.5) respectively. The CIP consolidation regimen has been a safety and well tolerated chemotherapy for all pts. Mortality-related chemotherapy occurred in 3 (3%) of the pts during P-VABEC. **Conclusions:** Our preliminary results showed that CIP consolidation therapy did not improve the survival of elderly NHL pts previously treated with P-VABEC. The study is still ongoing, additional pts and follow up will be need for definitive results.

## **ALLOGENEIC TRANSPLANTATION**

### **C082**

#### **HIGH VS. LOW DOSE OF POLYVALENT INTRAVENOUS IMMUNOGLOBULIN IN ALLOGENEIC HEMOPOIETIC STEM CELL TRANSPLANTS**

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The role of high dose intravenous IgG (HDIGG) in the Allogeneic Bone Marrow Transplantation setting is still under investigation, since there are contrasting clinical reports. In this study we compare two different doses of human HDIGG in a randomized prospective trial. Primary end points are infections, severity of acute graft versus host disease and transplant related mortality. Secondary end points are days of hospitalization, chronic graft versus host disease, days of intravenous antimicrobial and immunosuppressor therapy, incidence of transplant related TTP. Patients were randomised to received 100 mg/kg/week of HDIGG (Group A; n=38) or 400 mg/kg/week of HDIGG (Group B; n=37) from day -7 to day +100. The two groups were comparable for age, diagnosis disease status and acute graft-versus host prophylaxis. In spite of the different median concentration of seric IgG, we did not see significant differences between the two groups in terms of incidence of infections (local or disseminate), development of interstitial pneumonia (10 vs. 13), development of CMV infection (20 vs 24). Regarding acute GvHD, grade 0-I, II, III\_IV were observed in 22 vs 22, 14 vs 12 and 2 vs 3 patients respectively for the two arms. Chronic GvHD was absent in 5 vs 5, limited in 20 vs 19 and extensive in 13 vs 13. The days of hospitalization, of fever, of intravenous antibiotics, of intravenous immunosuppressive agents and the TTP index were not significantly different. Actuarial 1 year TRM is 16% vs 15% respectively. **Conclusions:** No differences were observed in two groups of patient receiving 100 or 400 mg/kg/week of

IgG. In view of the high cost of HDIGG, these results may have economic as well medical impact for allogeneic bone marrow transplant procedures.

### **C083**

#### **EXPRESSION OF ADHESION MOLECULES ON NORMAL CD34<sup>+</sup> STEM CELLS: A POSSIBLE ROLE IN MYELOID ENGRAFTMENT AFTER ALLOGENEIC TRANSPLANTATION**

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The expression of adhesion molecules on CD34<sup>+</sup> cells from healthy donors has been studied in order to evaluate their role in hematopoietic reconstitution after allogeneic stem cell transplantation (alloSCT). Twenty-two patients (pts.) affected by hematological diseases (4 MM, 5 MDS, 2 CML, 2 AML, 1 CLL, 3 NHL, 4 ALL, 1 SAA) were included in the study. Median age was 45 years (range 2-60); 13 pts. received peripheral blood stem cells (PBSC), 9 bone marrow (BM). The median number of CD34<sup>+</sup> cells infused was 2.8 x10<sup>6</sup>/kg (range 1.3-5.7) and 8.7 x10<sup>6</sup>/kg (range 2.8-15.6) for BM and PBSC recipients, respectively ( $P < .001$ ); the median number of CFU-GM was 10.2x10<sup>4</sup>/kg (range 0.9-33.6) and 56.3 x10<sup>4</sup>/kg (range 36-110) for BM and PBSC recipients, respectively ( $P < .001$ ). Recovery of PMN  $\geq 0.5 \times 10^9/L$  and PLT  $\geq 2.0 \times 10^9/L$  was not different among recipients of BM or PBSC graft. A three color flow cytometric assay was performed to study the expression of a large panel of integrins (CD49b, CD49d, CD49f, CD11a, CD11b, CD11c, CD18) and L-selectin (tested by Lecam and Leu8) on CD34<sup>+</sup> cells. A greater expression of CD49d, CD49f, CD11a, CD11b and CD18 was observed in BM compared to PBSC ( $P < .001$  for all comparisons), suggesting that G-CSF mobilises subsets of stem cells with lower adhesive properties. As for PMN recovery, subsets expressing Lecam, Leu8 and CD11c correlated better than total number of CD34<sup>+</sup> stem cells ( $r = -0.48$ ,  $r = -0.47$ ,  $r = -0.56$  vs.  $r = -0.41$ , respectively). Subsets expressing CD49d, Lecam, Leu8 and CD11c correlated with PLT recovery better than

total number of CD34<sup>+</sup> stem cells ( $r = -0.46$ ,  $r = -0.54$ ,  $r = -0.67$ ,  $r = -0.51$  vs.  $r = -0.42$ ). In a multivariate analysis, the number of LECAM<sup>+</sup>/CD34<sup>+</sup> cells approached the statistical significance for PLT engraftment ( $P = .052$ ). We conclude that the expression on CD34<sup>+</sup> stem cells of L-selectin and CD11c may identify subsets of hematopoietic precursors with unique biologic features promoting a faster myeloid recovery after alloSCT.

#### **C084 MONITORING OF MIXED CHIMERISM AFTER ALLOGENIC TRANSPLANTATION: MULTIPLEX AMPLIFICATION WITH FLUORIMETRIC DETECTION**

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We evaluated the feasibility and the sensitivity in detecting mixed chimerism after allogeneic transplantation of BM or PBSC using a commercially available kit (AmpF/STR Profiler Plus - PE Applied Biosystems) and ABI Prism 310 as detecting system. The multiplex amplification of 9 different short tandem repeats (STR) and of amelogenin locus is carried out in a single reaction tube. ABI Prism 310 detects the fluorescent signal and calculates the fragment size and the peak area. The sensitivity and linearity of this method has been tested on a serial dilution of whole blood and genomic DNA mixed in various proportions. The allele concentration in the 15 dilution samples tested ranged from 0.25 % to 99.75 %. The linearity of the response assessed in the interval 10 - 90 % showed a coefficient regression of 0.99. The mean sensitivity was 1.5 % (range 0.5 - 2.5), which was greater than that previously reported (Thiede et al. 1998, Scharf et al. 1995). In 25 donor-recipient couples the mean number of loci useful to assess relapse was 5.9 (range 3-11). We monitored 21 cases of allogeneic BM or PBSC transplantation (18 from family donor and 3 MUD) before, 1, 2 and at least 6 months from transplantation (range 1-24). In 6 patients mixed chimerism was observed, with a range of donor allele percentage ranging from 1-3 %. This percentage did not change over time and has not

been correlated with relapse. Fragment dimension reproducibility (cv 0.21, range 0.12-0.5) was confirmed either by quality control on a DNA sample either by amelogenin repeatability on all the patients. In conclusion, the present system is sufficiently rapid, sensible and specific for the evaluation of mixed chimerism after BM or PBSC transplantation.

#### **C085 CONVENTIONAL AND MOLECULAR CYTOGENETICS IN BONE MARROW TRANSPLANTED PATIENTS**

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We need to find more sensitive and specific methods for detecting and monitoring chimerism after allogeneic bone marrow transplantation (alloBMT). Conventional cytogenetic analysis allows to analyze only proliferating cells, and after alloBMT it could be difficult to get a sufficient number of good quality metaphases. Recently in sex mismatched alloBMT recipients, FISH analysis with sex chromosome probes (X-Y FISH) proved to be more sensitive and specific than conventional cytogenetics: X-Y FISH examines both proliferating and resting cells, thus giving information on the number of residual host cells, even if no metaphases are available. A technique which can be considered alternative to in situ hybridization could be the primed in situ labelling (PRINS), a method for labelling specific DNA sequences by annealing an oligonucleotide DNA primer to the denatured DNA on glass slides and extending it enzymatically in situ with the incorporation of labelled nucleotides. We set up this technique on both bone marrow and peripheral blood smears using X and Y primers in ten sex-mismatched transplanted patients. Preliminary data show a high specificity and the same sensitivity of FISH. However, this method is faster (about 2 hours) and approximately ten times less expensive than FISH.

## C086 SECONDARY FAILURE OF PLATELET RECOVERY IN HEMATOPOIETIC STEM CELL TRANSPLANTATION (HSCT)

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After HSCT, the recovery of platelet counts (PLT) is not always sustained, even though other cell lineages may return to normal ranges. The purpose of this study was to characterize secondary failure of platelet recovery (SFPR), defined as the first day of decline of PLT below 20000/ $\mu$ l, for 7 consecutive days or requiring transfusions within 7 days, after sustained platelet recovery (PLT  $\geq$ 50000/ml for 7 consecutive days without transfusion support) in a large series of patients undergoing HSCT. The study population consisted of 2871 consecutive patients transplanted at the FHCRC from Jan.'90 to Mar.'97. Patients were observed from the day of transplant until the first occurrence of relapse, second transplant, or discharge home. SFPR not due to disease recurrence was observed in 285/2153 (13%) of allogeneic patients and 36/718 (5%) of autologous patients, with a median time of onset at day 63 (range 21-156) and at 44 (range 24-89) days posttransplant, respectively. Concomitant neutropenia was present in 57/285 (20%) of allogeneic and 7/36 (19%) of autologous patients; 32/57 (56%) and 4/7 (57%) respectively, were on ganciclovir. By multivariate analysis, a transplant from an unrelated donor, GVHD prophylaxis other than methotrexate and cyclosporine, and time dependant variables such as development of grade II-IV GVHD, impaired renal and liver functions, and infections were highly significant risk factors. CMV infections were the only significant risk factor in the autologous setting. Baseline variables, known to correlate with platelet recovery, such as source of stem cells, cell dose infused, type of disease and disease status were not associated with a higher risk of developing SFPR. Most bone marrow aspirates performed on thrombocytopenic patients showed trilineage engraftment indicating defective thrombopoiesis or decreased platelet survival. One-year mortality was 51% in allotransplants and 44% in auto-

transplants with SFPR. The hazard of death was significantly higher in patients who experienced SFPR (HR=2.8 for allogeneic HSCT, HR 2.1 for autologous HSCT). SFPR is associated with adverse prognosis. A better understanding of the pathophysiology is warranted to design effective treatment strategies in a group of patients with poor outcome.

## C087 CTL-p ANALYSIS IN STEM CELLS TRANSPLANTATION (BMT) FROM HLA- IDENTICAL SIBLINGS

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In BMT setting, current matching policies are based on a "structural matching", i.e. donor-recipient identity for the HLA antigens or genes analysed. Nevertheless acute graft vs host disease (aGvHD) occurs even in the presence of a complete "structural matching", whereas sometime recipients from a known mismatched donor do not develop significant aGvHD. The definition of a "functional matching" may help to assess the clinical relevance of mismatches for known HLA antigens, minor histocompatibility antigens (mHA) or undefined antigens not detected by current methods. The precursor frequencies of donor antirecipient cytotoxic T-lymphocytes (fCTL-p) were shown to predict the occurrence of GvHD after BMT from unrelated donors. In the present study, the CTL-p frequency and the incidence and severity of aGvHD in 42 BMT from HLA-identical sibling for onco-hematological diseases have been compared, in order to assess the value of CTL-p assay in donor selection and possibly in the graduation of GvHD prophylaxis. Pre-BMT conditioning was TBI-CY in 21 pts., Thiotepa-CY in 14 and Bu-CY4 in 7. GvHD prophylaxis consisted of CyA + MTX in all cases. Sibling donors were selected on the basis of HLA identity using serological typing for HLA-A,B,C antigens, whereas HLA-DRB, DQA, DQB was tested by molecular analysis. All CTL-p assays were performed in the GvH direction, with donor cells as responders and recipient cells as stimulators. Donor/recipients pairs were divided into high

(>1/100.000) and low (<1/100.000) CTL-p frequency groups. 13/18(72%) patients in the high frequency group and 10/24 (42%) in the low frequency group experienced significant (II-IV) aGvHD. A significant correlation ( $p=0.048$ ) between CTL-p frequency and severity of aGvHD was demonstrated. The two groups of pts were compared with regard to other clinical features: patient and donor age, time to engraftment, patient and donor sex, time to aGvHD onset, relapse, outcome and CMV status. No significant differences were detected. Even if these data are to be confirmed in a larger cohort of pts., in our experience the CTL-p assay provides useful information for predicting the severity of aGvHD in BMT from HLA-identical sibling.

### C088

#### AGVHD WITH DONOR COMPLETE CHIMERISM AFTER CR OBTAINED WITH CONVENTIONAL CHEMOTHERAPY FOR RELAPSED ACUTE LEUKEMIA AFTER ALLOGENEIC TRANSPLANT

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The association of GVHD with GVL is a well known phenomenon suggested by higher incidence of relapse in patients (pts) not experiencing GVHD, in TCD transplant and in syngeneic transplant. On the other hand DLI can induce remission in relapsed pts. We describe here the case history of three pts affected by acute leukemia who relapsed after allogeneic transplantation; treated with conventional chemotherapy they obtained CR followed by the arise of AGVHD associated with disease-free survival in the presence of a full donor chimerism. **Clinical Characteristics.** Case 1: UPN 265, M, 29 years-old, ALL(Ph1+), 1CR, underwent MUD BMT, sex-mm, conditioning ifTBI 1320cGy+Cy 120, MTX+CSA+ATG for GVHD-prophylaxis, GVHD grade = 0, relapse on +206, IDA+ARA-C as reinduction chemotherapy, and CR. Case 2 UPN 385, F, 41 years old, ALL preT slow responder, conditioning ifTBI 1320cGy+Cy 120, MTX+CSA for GVHD-prophylaxis, GVHD grade = 0, relapse on +184, Vindesine and Dexame-

tazone as reinduction chemotherapy, and CR. Case 3 UPN 386, F, 41 years old, AML M4, conditioning Thiotepa 15+Cy 120, MTX+CSA for GVHD-prophylaxis, GVHD grade = 0, relapse on +223, FLAG as reinduction chemotherapy, and CR. After CR was reached in all cases a AGVHD arised and the evaluation of chimerism showed a fully donor marrow. The characteristics of clinical behaviour after CR are reported in the table.

	AGVHD	CHIMERISM	DFS	STATUS
<b>Case 1</b>	IV Liver	CC(46XX,Ph1-)	21m	Dead(cGVHD)
<b>Case 2</b>	III Skin and Liver	CC(VNTR)	+17	Alive (cGVHD)
<b>Case 3</b>	IVLiver	CC(46XY)	+6	Alive(cGVHD)

We hypotize that conventional chemotherapy allowed a proliferative advantage for the residual donor hematopoiesis, triggering the GVHD and possibly a GVL effect with maintained CR in pts with very aggressive disease relapsed after allogeneic transplant.

### C089

#### BENEFICIAL NK CELL ALLOREACTIONS IN MISMATCHED HEMATOPOIETIC TRANSPLANTS

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Full haplotype-mismatched hematopoietic stem cell transplants have recently been employed for treatment of bad-risk leukemia patients lacking a matched donor (N Engl J Med, 339:1186, 1998). Because of the expression of inhibitory receptors (KIR) for epitopes shared by certain MHC class I allotypes, a person's NK cells will not recognize and will, therefore, kill cells from individuals lacking his/her KIR epitope. Although KIR epitope mis-matches are well known causes of NK cell alloreactivity, their role in human transplantation have not been evaluated. We investigated the role of the three major NK specificities, i.e., those for HLA-C group 1, HLA-C group 2, and HLA-Bw4 alleles. In 20/61 donor-recipient pairs, KIR epitope incompatibility and functional analyses of donor NK cell clones predicted donor NK cells could cause GvH/GvL reac-

tions. NK cell clones of donor origin were obtained from transplanted recipients and tested for lysis of recipient's cryopreserved pre-transplant lymphocytes. Despite the absence of GvHD, we detected high frequencies of NK clones which killed recipient's target cells. Lysis followed the rules of NK cell alloreactivity, being blocked only by the MHC class I KIR epitope which was missing in the recipient. The alloreactive NK clones also killed 100% of the allogeneic myeloid leukemias tested (and only a minority of ALLs). Therefore, potential *in vivo* targets are myeloid leukemias, as they were susceptible to allogeneic NK killing *in vitro*, but also host lymphocytes mediating rejection, because assessment of allogeneic NK lysis was routinely performed against activated lymphocytes. No myeloid relapses or graft rejections were observed in the 20 patients transplanted from donors with HLA-based potential for transfer of anti-recipient NK cell alloreactivity. To date the 5 myeloid relapses and the 7 rejections have occurred in the other 41 patients. In conclusion, the present study uncovers one biological aspect of mismatched hematopoietic transplantation, i.e., the unexpected post-grafting emergence of donor NK cells which do not recognize host alloantigens and which, in the absence of GvHD, kill recipient target cells in accordance with the laws of NK cell alloreactivity. KIR epitope-mismatching in the GvH direction may confer unique potential for GvL effect and for engraftment (Blood 1999, *in press*).

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## **C090 ENGRAFTMENT IN NOD/SCID MICE OF HUMAN CORD BLOOD CD34<sup>+</sup> CELLS AFTER EX VIVO EXPANSION**

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Although cord blood (CB) compares favourably to other hemopoietic stem cell sources in regard to a number of parameters, its use in large patients is limited by

the low number of cells that are available. Ex vivo expansion of CB stem cells might be used to overcome this limitation. We have previously reported that CD34<sup>+</sup> CB cells can be expanded *in vitro*, for several months, in stroma free cultures in the presence of FL+TPO±IL-6±SCF. Recently a new approach has been developed to establish an *in vivo* model for human primitive hematopoietic precursors by transplanting human hematopoietic cells into sublethally irradiated NOD/SCID mice.

We have examined the expansion of SCID-Repopulating Cells (SRCs) during stroma free suspension cultures of human CD34<sup>+</sup> CB cells. Groups of sublethally irradiated NOD/SCID mice were injected with 10,000 and 20,000 unmanipulated CD34<sup>+</sup> CB cells which were cryopreserved at the start of cultures, either the cryopreserved corresponding progeny of initial 10,000 or 20,000 CD34<sup>+</sup> cells that were cultured for 4, 8 and 12 weeks in presence of FL, MGDF, SCF and IL-6. Mice were sacrificed 6-8 weeks post transplant and BM and spleen were assessed for human engraftment by flow cytometry, DNA analysis, growth of human myeloid and erythroid progenitors and LTC-IC. These techniques reliably detect human cells at very low levels in a murine background. Mice that had been injected with 10,000 or 20,000 cryopreserved uncultured CD34<sup>+</sup> cells did not show any sign of engraftment. Conversely, mice injected with the cryopreserved and expanded cells generated by 10,000 or 20,000 initial CD34<sup>+</sup> cells showed a good level of engraftment. These results support and extend our previous findings that CD34<sup>+</sup> CB stem cells (identified as LTC-IC) could indeed be grown and expanded *in vitro* for an extremely long period of time.