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ABSTRACT BOOK

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MAIN PROGRAM

INTEGRATED DIAGNOSTICS OF AML: FROM MORPHOLOGY TO NEXT GENERATION SEQUENCING

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The diagnostic approach to AML experienced several breathtaking changes over the last decade. However, still methods known for more than 100 years are the primary backbone of the diagnostic approach: cytomorphology including cytochemistry. These techniques can be applied very quick (1-3 hours), give a lot of important information for the diagnosis of the disease and especially are gatekeepers for other techniques that have to be applied in parallel and are more and more specific but also labour- and cost-intensive.

The second mandatory technique is standard cytogenetics based on chromosome banding analysis. Even today, the information taken from this investigation gives the most important prognostic marker in AML and helps to stratify patients into several risk adapted groups including treatment stratification. However, metaphase cytogenetics needs 3-7 days for results and is (as well as cytomorphology) dependant on very skilled and experienced personnel. Central reference laboratories with expertise and experience need to take over the responsibility for cytogenetics as quality control rounds clearly demonstrate that the experience of the investigators is a very important factor to come to quick, reliable and patient-orientated results.

In parallel to cytomorphology, immunophenotyping is still a method of choice to diagnose AML, especially very immature AML (formerly called FAB M0) and megakaryoblastic leukemia (formerly called FAB M7). Immunophenotyping can also help to pick up treatment related expression profiles such as CD33 and also delivers markers for minimal residual disease measurement (leukemia associated immunophenotype, LAIP).

In addition to cytomorphology, cytogenetics and immunophenotyping in the last ten years molecular genetics revolutionized the characterization, classification and prognostication of AML. This is especially true for patients with a normal karyotype (45% of all AML patients). Therefore, an increasing number of genes demonstrating mutations in AML have to be investigated to fully characterize patients' disease and especially patients' risk. This has an important implication even for transplantation strategies and lead more and more to a "target-specific" indivudual approaches also in AML based on these molecular markers.

Today it seems necessary to at least investigate *FLT3*-ITD, *CEBPA* and *NPM1* to describe patients' risk especially in normal karyotype. All these three markers can also be used for minimal residual disease follow-up studies, especially *NPM1* has been established to give important information. On the other hand, genes such as *MLL*-PTD, *RUNX1, ASXL1* and genes recently detected by next generation sequencing such as *DNMT3A, IDH1, IDH2,* or *BCOR* have been shown to discriminate patients' risk in at least some subgroups of patients. Also T*P53* has potential power to better define risk in AML.

By looking at the whole portfolio of mutations that have been depicted in the last ten years in AML it is clear that – as combinations of several markers are possible – not for all possible scenarios prognostic information or treatment stratification is available. However, without investigating all these markers in parallel, this information will never be available and so the trials that are performed in AML and especially those trials that will start in AML have to investigate all these markers by new approaches of laboratory work-up. This said, next generation sequencing (NGS) will take over very soon the broad spectrum of molecular investigations in AML. It is foreseeable that in the near future, molecular investigations in AML will be bundled to gene panels and then measured on next generation sequencing platforms in a very short time, i.e. below 3-4 days per patient.

In conclusion, the diagnosis if AML is still based on morphology, because it is quick and cheap and can guide the other techniques, cytogenetics and immunophenotyping. The broad spectrum of molecular markers has to be investigated in parallel to use its important information prospectively. To make this possible, laboratory workflows have to be rescheduled quickly and next generation sequencing will take over a lot of responsibilities from standard sequencing today. AML is one of the best examples to implement these new workflows nad instruments and can be used as a paradigm for many other diseases in hematology and oncology in the next decade: for diagnosis, prognostication and treatment stratification.

TARGETING THE MTOR PATHWAY IN AML

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Despite recent advances in the field, the treatment of acute myeloid leukemia (AML) remains challenging. Although complete remissions can be achieved in the majority of patients treated with intensive chemotherapy, many of them will relapse and eventually succumb to their disease. Meanwhile, much new molecular knowledge is emerging, which could both guide treatment decisions and provide opportunities for more effective and less toxic novel therapies.[1]

The major thrust of novel therapeutics in AML is development of agents targeting critical cellular and molecular events that ultimately lead to leukemic transformation. One promising target for molecular therapy in AML is the PI3K/Akt signalling pathway, which is constitutively activated in AML samples and has been shown to be central to the proliferation and survival of the leukemic blasts.[2] The serine-threonine kinase mTOR is downstream of PI3K/Akt and can be inhibited by selective inhibitors including rapamycin (sirolimus) and its first generation analogues (rapalogs). mTOR inhibitors have been shown to induce apoptosis of AML cells in vitro, to have in vivo activity in experimental models of AML, and to enhance the antileukemic activity of a variety of cytotoxic agents including etoposide, anthracyclines and cytarabine.[3]

Clinical trials of rapalogs are ongoing in AML as single agents and in combination with chemotherapy. However, despite promising evidence of biological on target effects, rapalogs have proven only modestly successful.[4-7] Clearly, this may be a reflection of the limited biologic activity of rapamycin/rapalogs as these agents are known to inhibit mTORC1 but not, as a general rule, the rapamycin-insensitive mTORC2 functional complex. This differential inhibitory effect can lead to a paradoxical activation of pro-survival signaling pathways upstream of mTOR including those mediated by PI3K and Akt, ultimately resulting in rapamycin resistance.[8] Whether this preclinical findings are of clinical relevance is still unknown, but much effort is being made to develop a new generation of agents targeting the PI3K/Akt/mTOR network at multiple sites.[9] Inhibitors targeting both mTORC1 and mTORC2 (ATP-competitive catalytic site inhibitors), as well as dual catalytic PI3K/mTOR inhibitors are currently undergoing early phase clinical testing, and it is hoped that they will lead to more optimal targeting of this pathway for AML therapy.[10,11]

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DIAMOND BLACKFAN ANAEMIA: A RIBOSOMOPATHY

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DBA is an inherited red cell aplasia, that shows autosomal dominant transmission. It is characterized by severe normochromic-macrocytic anemia often associated with malformations. Although it is rare (frequency: 6/million live births), it holds an important place in hematology as a paradigm of an intrinsic genetic disorder of the committed erythroid progenitor. Bone marrow is normocellular with a selective deficiency of red cell precursors. Proliferative and differentiative activity towards the other lineages is normal. Defective erythropoiesis is shown in the bone marrow by a very low number of erythroid precursors, and functionally by a reduction of BFU–E progenitor cells.[1] DBA patients display high EPO levels, irrespective of the degree of their anemia. The failure of their hemopoietic progenitors to respond to EPO suggests EPO insensitivity. More than 50% of patients respond to steroid therapy. About 10% of patients undergo disease remission, but its causes are unknown. Options in steroid–resistant patients are chronic red cell transfusions or allogenic stem cell transplantation.[2] Life expectancy is reduced and some patients develop malignancies.[3]

An international collaborative group to which we belong identified the first DBA locus on 19q13.2, but also locus heterogeneity.[4,5] This locus encodes for a structural ribosomal protein (RP), RPS19, the first RP known to cause a human disease .[6] RPS19 mutations are found in 25% of patients.[7-10] Mutations have been identified in 8 other RP genes: RPS24, RPS17, RPS7, RPL5, RPL11, RPL35A, RPS26, RPS10.[11-16] GATA1 mutations have been identified in rare patients.[17] We have set up a DBA Gene Mutation Database, that classifies all DBA mutations.[18,19]

Diagnosis is made after exclusion of other bone marrow failure syndromes (BMFS). Most DBA patients show high levels of erythrocyte adenosine deaminase. Confirmation of diagnosis relies on finding mutations in DBA genes. Methods like MLPA or CGH array should be employed to identify large deletions.[20,21])

The mechanisms that lead to defective erythropoiesis have not been completely elucidated. The defect in RPs either of the small or the large subunit suggested that the mechanism leading to BMF is due to a defect of ribosome biogenesis.[22-23] Abnormal rRNA maturation and defect in polysomes and/or in ribosomal subunits have been reported in yeast and human cells downregulated for various RPs,[24,13] in CD34+/– cells[25] and lymphocytes[26] from DBA patients. Other inherited BMFS are due to defects in genes involved in ribosome function.[27]

Slightly decreased erythropoiesis was shown by zebrafish with RP defects[28-30] and by a mouse carrying a missense mutations of RPS19,[31] whereas Ko mice for RPS19 on both alleles die prior to implantation and heterozygous mice have a normal phenotype.[32] Macrocytic anemia was shown by a mouse model for RPS19-deficient DBA using transgenic RNA interference that allows an inducible and graded downregulation of Rps19.[33] A current model to explain the pathogenesis of DBA proposes that the abnormal ribosome biogenesis triggers apoptosis of erythroid precursors through activation of a specific ribosomal stress (RS). Activation of p53 in response to RS has been shown in model organisms, cultured cells and patient bone marrows.[34-36,26] Using primary human progenitors cells after partial knockdown of RPS14 and RPS19 Dutt et al. have shown that erythroid committed cells are more sensitive to p53 activation as compared to other hemopoietic lineages. Such activation may be due to an increase in free RPs, that interact with MDM2 suppressing its ubiquitination of p53. So p53 is not targeted to degradation.[36] However, p53-independent pathways, activated in response to RS, have also been suggested.[34] Erythroid cell lines such as TF1, that carry p53 inactivating mutations, show reduced proliferation, abnormal expression of proapoptotic genes and genes involved in protein synthesis when a RP is downregulated.[37] A peculiar translation pattern has also been reported in mice cells silenced for RPS19 and RPL11.[38] Thus, cells with RS develop a specific transcriptional and translational pattern that may be at least partially responsible for the erythroid defect.

The possibility to counteract the activation of the RS response without direct modification of the defective gene is suggested by several studies. Aneddotical reports suggested the use of leucine supplementation. Lenalidomide treatment has been proposed, because it is able to attenuate the erythropoietic failure in 5q-myelodysplasia. A subset of 5q- myelodysplasias are due to deletion of RPS14. In vitro and in vivo studies showed that p53 inhibition may attenuate the erythroid proliferation and maturation defect, but it cannot be proposed as a treatment. A better definition of the mechanisms that activate or attenuate RS are mandatory and may identify new therapeutic solutions.

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CONGENITAL DYSERYTHROPOIETIC ANAEMIA (CDA) TYPE II: AN ALMOST REVEALED MISTERY

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Congenital dyserythropoietic anaemias (CDA) represent a heterogeneous group of rare disorders characterized by anaemia and marked functional and morphological alterations of erythropoiesis. In 1968, Heimpel and Wendt proposed a classification of CDA into three major subtypes, I, II and III (Heimpel & Wendt, 1968; Wickramasinghe 1997). Of these, CDA II is the most common, and is characterized by autosomic recessive inheritance, normocytic erythrocytes, bi- or multinucleated erythroblasts, and the presence of a double plasma membrane, which originates from the endoplasmic reticulum (Iolascon et al., 1996). More than 500 cases of CDA II have been described, especially in Northern Europe, Northern Africa and Italy (Heimpel et al., 2010). CDA II represents the most frequent form of CDA with prevalence varying between European regions, with minimal values of 0.08 cases/ million in Scandinavia and 2.60 cases/million in Italy [Heimpel et al.,2010].

The diagnosis of CDA II is usually made in childhood, but the severity of the disease varies from mild to severe anaemia requiring chronic transfusion therapy. Anemia is usually accompanied by jaundice, hepatosplenomegaly, worsening of anaemia with intercurrent infections, and gallstones. Progressive iron overload is also associated with CDA II, probably as a consequence of increased intestinal absorption (Cazzola et al., 1983).

Some cases of CDA II have been linked to a gene on the long arm of chromosome 20 (20q 11.2), but the disease seems to be genetically heterogeneous (Gasparini et al., 1997; Iolascon et al., 1997, 1998). The true incidence of CDA II is probably underestimated due to the presence of only mild anaemia in many cases and to being misdiagnosed as hereditary spherocytosis (HS). In fact, both diseases may present with anaemia, jaundice, splenomegaly, increased osmotic fragility, and presence of microspherocytes on the peripheral smear. Although splenectomy is effective in reducing anaemia in both CDA II and HS, iron overload is common in CDA II and very rare in HS. For this reason, correct identification of CDA II leads to appropriate preventive and therapeutic measures to avoid the organ damage associated with iron overload.

Using some cases from consanguineous families, K. Schwartz in collaboration with our group was able to redefine the region containing CDA II locus in a new interval, between D20S112-D20S106 markers: it contained 125 genes. Too much genes to solve this problem using the sequencing approach. In order to narrow down this region, we performed microarray analysis on RNA from $CD34^+$ to orthochromatic normoblasts (14-21 days after erythroid differentiation by EPO) of healthy controls to identify differentially expressed genes during normal erythropoiesis. Supposing that CDA II was due to the absence or reduction of a protein, all up-regulated genes mapped in this area were examined. Based on this information, we selected 11 genes. With the assumption that the *cis*, median and *trans* N-glycan Golgi processing of erythroblasts glycoproteins was impaired, the *SEC23B* gene became a likely candidate. When we started to sequence it, immediately we demonstrated a lot of different mutations in Italian, French and German cases [Schwartz K et al. 2009].

To clarify the function of *SEC23B* gene in erythrocyte development, we examined its role in the zebrafish embryo by injection of antisense morpholinos. In morphants, erythrocytes look immature and a significant increase in immature, binucleated erythrocytes was detected.[10] In human cellular model we elucidated the expression of SEC23B in erythropoietic lineage during differentiation. During this period there is a clear up-load of SEC23B whereas the counterpart SEC23A is down-regulated [Schwartz K et al. 2009]. Gene *SEC23B* encodes the SEC23B component which is part of the cytoplasmic coat protein (COP)II complex [Barlowe C et al, 1994] that controls the protein trafficking from RE and Golgi.

Up to now, 65 CDA II unrelated cases have been described [Schwartz K et al.,2009,Biamchi P et al, 2010, Iolascon A et al. 2010; Fermo E et al, 2010], with a lot of different causative mutations . In a preliminary way we could conclude that any part of the gene could be mutated and that in the vast majority of countries there are sporadic mutations. The higher incidence of CDA II in Italy prompted us to search for the existence of a founder effect, particularly in southern Italy. There are at least four mutations appearing more frequent in southern Italy. This information could be relevant for the diagnosis of new cases.

Recently we identified an array of mutations in the *SEC23B* gene within a cohort of 42 unrelated CDA II patients originating mostly from Italy and from France, but also from other parts of the world [Iolascon A et al, 2010: Russo R et al, 2011]. Correlation between the mutations and various biological parameters suggested that the association of one missense mutation and one nonsense mutation was significantly more deleterious that the association of two missense mutations; or rather, there is a trend for patients carrying two missense mutations to be more mildly affected. Nevertheless, there remained an obvious overlap between the two groups. Homozygosity for two nonsense mutations was never encountered and must be fatal[Iolascon A et al.2010].

The pathogenesis of CDA II seems due to abnormal vesicular transport ER-Golgi. We could hypothesize that this transport involves a specific group of proteins implicated in cytokinesis, and this could explain the basis of morphological abnormality (dyserythropoiesis). However, the effective relationship between mutations in *SEC23B* gene and ineffective erythropoiesis is not yet completely understood.

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ROLE OF MORPHOLOGY IN HEMATOLOGY DIAGNOSIS TODAY.

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Among medical specialties, hematology is a relatively young but rapidly expanding discipline with peculiar innovative and multidisciplinary aspects. The strict connections with cytogenetics, molecular biology, immunology, transplant, surgery and bioethics generate widespread data and scientific publications. Many factors contribute to the substantial annual increase of hematological patients. One of this is associate with the continuously increasing mean life expectancy in Western Countries, that means an increase in the absolute number of newly diagnosed age-linked hemopathologies. Key factors are represented by research programs that lead to the identification of new molecular markers for diagnosis and prognostic stratification and screening programs that lead to early diagnosis and longer patients' follow-up and survival through the use of new diagnostic and therapeutic technologies.

Classical diagnostic hematological pathway concerns the identification and classification of cells derived from bone marrow and peripheral blood according to all international diagnostic protocols such as FAB 1982 and 1986, MIC 1989 REAL 1990, WHO2000 and WHO 2008. Morphological evaluation of peripheral blood (PB) and bone marrow (BM) cells through microscopic examination of properly stained smears remains crucial in hematological diagnosis. Many factors, such as differences in bone marrow processing procedures, staining, degree of skill in interpretation and terminology used, contribute to a lack of standardization of this diagnostic tool. However, still in the new WHO classification of Hematological malignancies it is highlighted the importance of morphological aspects, quantitative as well as qualitative, for the recognition of disease entities and better stratification of patients with hematological neoplasms. According to the international rules for the diagnosis in hematology it is mandatory the examination and the identification of 200 consecutive cells from PB smears for the report of the Differential and 500 consecutive cells from BM smears, for the report of the myelogram. Moreover to detect some highly pathological morphological aspects, such as dysplasia in the cells, it is mandatory to examine at least 200 cells from each lineage, myeloid and erythroid. In conclusion evaluation of blood and bone marrow cytology is a stakeholder step as well the first one in the integrated diagnostic process of hematological diseases. Moreover the discovery of peculiar morphologic features associated with specific genetics/molecular genetics abnormalities makes the skilful microscope evaluation fundamental for the correct diagnostic pathway saving time and resources.

To reach an harmonized morphological diagnosis international scientific working groups and morphological committees have to work by exchanging same slides each other, matching the final reports and discussing discrepancies in order to reach a final consensus morphological diagnosis. The current information and communication technology (ICT) era provides the opportunity to exchange, via internet, images and information without geographic limitation. In the field of hematology, many studies highlight the robustness of ICT for diagnostic assessment of blood cells. ICT is therefore well suited to pursue a full consensus in the assessment of cell morphology. The develop of a new technologic tool based on the scan of the whole smear open new and realistic opportunities: putting in the web the scan of the smears of PB and BM all the virtual community involved in this knowledge process can be trained adopting the same diagnostic procedures as for those adopted in the real life for the diagnosis at microscope of hematological patients.

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MOLECULAR BASIS FOR A TARGETED THERAPY IN HAIRY CELL LEUKEMIA

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Hairy Cell Leukemia (HCL) has distinctive clinico-pathological features among mature chronic B-cell leukemias, including unique immunophenotype (*e.g.*, co-expression of CD11c, CD103, CD25 and Annexin-1) and genome-wide expression profile (resembling memory B cells), as well as exquisite sensitivity to chemotherapy with the purine analogs cladribine and pentostatin.

However, the genetic basis underlying HCL has been unknown until we recently discovered, by whole-exome sequencing of leukemic and normal cells purified from one HCL patient, that an activating mutation (V600E) of the BRAF kinase is the disease-defining genetic lesion of HCL (Tiacci et al., New Engl J Med 2011;364:2305), similar to BCR-ABL1 in chronic myeloid leukemia. In fact, the BRAF-V600E mutation is present in virtually 100% of almost 500 HCL patients from all over the world we and others have analyzed. Conversely, this mutation is absent in virtually all 300 patients analyzed with HCL-like disorders (such as splenic marginal zone lymphoma and HCL-variant), that have a similar clinico-pathological picture but a worse prognosis and that require a different therapeutic approach. To help in this differential diagnosis, we have also developed a simple PCR test to detect the BRAF-V600E mutation with high sensitivity and non-invasively in whole blood samples (Tiacci et al., Blood 2012;119:192).

The BRAF-V600E mutation leads to constitutive activation of the oncogenic MEK-ERK pathway in solid tumors harboring this mutation. We observed that, *in vitro*, pharmacological inhibition of BRAF-V600E in primary HCL cells causes MEK and ERK dephosphorylation, followed by loss of the hairy morphology and eventually apoptosis, establishing a strong pre-clinical therapeutic rationale for inhibiting the BRAF-MEK-ERK cascade in HCL. Indeed, small-molecule oral inhibitors exist that specifically target the mutated BRAF kinase or the MEK kinase (the BRAF downstream target) and that have shown remarkable clinical activity in metastatic melanoma patients harboring the BRAF-V600E mutation. Therefore, this mutation and the consequent MEK/ERK phosphorylation represent an important new target in HCL, whose detection is becoming increasingly relevant from a diagnostic standpoint and it is likely to become so also from a therapeutic perspective.

NUCLEOTIDE-METABOLIZING ENZYMES AND TUMOR-HOST INTERACTIONS IN CHRONIC LYMPHOPROLIFERATIVE DISEASES

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Nicotinamide adenine dinucleotide (NAD) is an essential co-enzyme that can be released in the extracellular milieu. Here, it may elicit signals through binding purinergic receptors. Alternatively, NAD may be dismantled to adenosine, in turn up-taken by cells and transformed to reconstitute the intracellular nucleotide pool. An articulated ecto-enzyme network is responsible for the nucleotide-nucleoside conversion. CD38 is the main mammalian enzyme that hydrolyzes NAD, generating Ca^{2+}-active metabolites. Evidence suggests that this extracellular network may be altered or used by tumor cells to i) nestle in protected areas, and ii) evade the immune response. We have exploited chronic lymphocytic leukemia as model to test the role of the ecto-enzyme network, starting to analyze the individual elements making up the whole picture.

MECHANISMS OF CHEMOREFRACTORINESS IN CHRONIC LYMPHOCYTIC LEUKEMIA: BEYOND TP53

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Although modern treatment strategies are highly effective in most chronic lymphocytic leukemia (CLL) patients, a challenging subgroup of cases show poor response to standard regimens and a survival of less than 2 years. In an optimized management algorithm of CLL, the early identification of chemorefractory patients, ideally prior to treatment, is a pre-requisite for designing strategies tailored at overcoming therapy resistance.[1] Fludarabine-refractoriness is due to *TP53* disruption by mutations and/or deletion of the locus in ~40% of CLL patients failing treatment (Figure 1A), but the molecular basis of this aggressive clinical phenotype remains unclear in the remaining sizeable fraction of patients.[2]

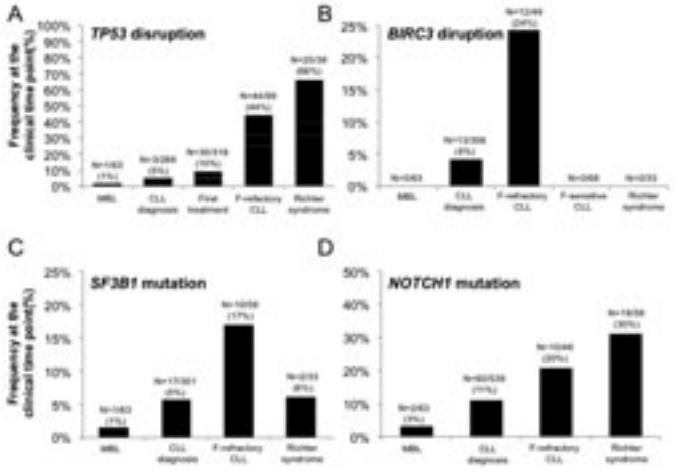

Figure 1. Notch1 mutations in Richter Syndrome and chemorefractory CLL.

The application of whole genome/exome sequencing to CLL has allowed to identify of a number of previously unrecognized and recurrently mutated genes in chemorefractory CLL, including *BIRC3*, *SF3B1*, and *NOTCH1*.[3-11]

Activation of the NF-kB pathway in CLL is regarded as a mechanism of resistance to disease eradication.[12-15] Specific interactions between protective microenvironmental niches and CLL cells activate NF-kB signaling, which in turn provides pro-survival signals to the leukemic clone through the upregulation of a number of anti-apoptotic genes.[12-15] Importantly, NF-kB activation correlates with CLL outcome and enhanced resistance to fludarabine.[12-15] At least in a fraction of cases, CLL cells might become independent from microenvironmental interactions by gaining active NF-kB signaling through the acquisition of functionally relevant mutations targeting NF-kB genes.[3,4,7] BIRC3 cooperates in a protein complex that negatively regulates MAP3K14, the central activator of non-canonical NF-kB signaling.[16] *BIRC3* is disrupted by truncating mutations, deletions, or a combination of both mutations and deletions in ~25% chemorefractory CLL (Figure 1B).[10] At the biochemical level, inactivating mutations of *BIRC3* cause the truncation of the C-terminal RING domain of the BIRC3 protein, whose E3 ubiquitin ligase activity is essential for switching off MAP3K14 through proteosomal degradation, thus leading to constitutive NF-kB activation.[10]

Splicing of pre-mRNA and formation of mature mRNA is carried out by the spliceosome, a complex of five small nuclear ribonucleoproteins (snRNPs).[17] *SF3B1* is a core component of the U2 snRNP, which recognizes the 3 splice site at the intron-exon junctions.[17] *SF3B1* mutations occur in ~20% fludarabine-refractory CLL (Figure 1C), and are generally represented by missense nucleotide changes clustering in selected HEAT repeats of the SF3B1 protein and recurrently targeting three hotspots (codons 662, 666 and 700), with a single amino-acid substitution (K700E) accounting for ~60% of all *SF3B1* mutations.[6] Though the precise biological role of *SF3B1* mutations in CLL is currently unknown, the clustering of mutations within structurally critical HEAT domains suggests that they are selected to modify *SF3B1* interactions with other proteins of the spliceosome complex.[5-7]

NOTCH1 encodes a class I transmembrane protein functioning as a ligand-activated transcription factor and playing an important role in several cellular process including apoptosis.[18] Upon ligand binding, the NOTCH1 intracellular domain translocates to the nucleus, thus leading to transcriptional activation of multiple target genes and pathways.[18] *NOTCH1* mutations in fludarabine-refractory CLL are frameshift or nonsense events, cluster within exon 34, and are mostly represented by one single 2-bp deletion (c.7544_7545delCT) that accounts for ~80% of all *NOTCH1* mutations.[3,9] The predicted functional consequence of *NOTCH1* mutations is the disruption of the C-terminal PEST domain of the NOTCH1 protein, which is physiologically required to limit the intensity and duration of NOTCH1 signaling. Removal of the PEST domain results in NOTCH1 impaired degradation and accumulation of an active NOTCH1 isoform sustaining deregulated signaling.[18] *NOTCH1* mutations recur in ~20% chemorefractory CLL (Figure 1D).[3,9] The functional relevance of *NOTCH1* mutations in the pathobiology of CLL resistance is reinforced by the finding that active *NOTCH1* signaling in this leukemia confers resistance to apoptosis in CLL cells through NF-kB activation.[18]

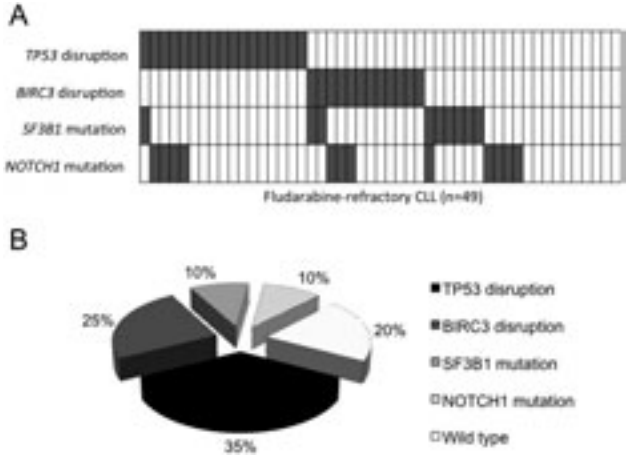

Figure 2.

Overall, *TP53*, *BIRC3*, *SF3B1*, and *NOTCH1* mutations distribute in a mutually exclusive fashion in chemorefractory CLL, suggesting that they are alternative mechanisms contributing to chemorefractoriness (Figure 2A), and overall account for ~80% cases of this highly aggressive clinical phenotype (Figure 2B).

From a clinical standpoint, *BIRC3*, *SF3B1*, and *NOTCH1* abnormalities may represent new biomarkers for the identification of poor risk patients already in the early phases of the disease. Consistent with this notion, among newly diagnosed CLL, *BIRC3*, *SF3B1*, and *NOTCH1* abnormalities identify a subgroup of high risk patients characterized by a poor outcome not different from that marked by *TP53* abnormalities (Table 1).[3-10] In addition, *BIRC3*, *NOTCH1*, and *SF3B1* abnormalities reveal novel and attractive candidate therapeutic targets in chemorefractory CLL, since NF-kB, NOTCH, and spliceosome inhibitors are already available,[18-20] and others are under active clinical development.

Table 1. Overall survival from diagnosis in CLL harboring TP53, BIRC3, SF3B1 and NOTCH1 lesions.

	MEDIAN OVERALL SURVIVAL
TP53 disruption	4.6 years
BIRC3 disruption	3.1 years
SF3B1 mutations	2.5 years
NOTCH1 mutations	3.5 years

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EPIGENETIC ALTERATIONS IN MYELODYSPLASTIC SYNDROMES

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Myelodysplastic Syndromes (MDS) are a heterogeneous group of neoplastic disorders characterized by bone marrow failure, uni- or multi-lineage dysplasia, impaired differentiation and an increased risk of transformation into acute myeloid leukemia (AML). Several factors contribute to the pathogenesis of MDS and evolution into AML and among these, the acquisition and accumulation of genetic and epigenetic changes play an essential role. Among epigenetic processes, DNA methylation physiologically regulates normal embryogenesis and development. It is mediated by DNA methyltransferases (DNMTs), which catalyse the conversion of cytosine residues located at CpG dinucleotides to 5-methylcytosine. DNMT1 has both maintenance and de novo methyltransferase activity, whereas DNMT3A and 3B are de novo methyltransferases. Histone code modifications, such as acetylation, methylation, ubiquitylation, phosphorylation, sumoylation and ADP-ribosylation modify the amino-terminal tails of histones protruding from the nucleosome. The acetylation of lysine residues on histones by histone acetyltransferases (HATs) causes the chromatin unfold leading to activation of transcription. On the other hand, the histone deacetyltransferases (HDACs) induces a tight compaction of the chromatin resulting in transcriptional gene repression. Besides these two classic epigenetic modifications, further epigenetic mechanisms have gained attention including miRNA regulation, Polycomb group (PCG) marks gene silencing and their interaction with transcription factors.[1-3]

In MDS, starting from the early 1990s hypermethylation of promoter regions of tumor suppressor genes (TSGs) was recognized as one of the leading transforming events, cooperating with genetic alterations. Many fundamental components of key cellular pathways were shown to be inactivated by hypermethylation in MDS and AML, including DNA repair (MGMT, BRCA1), cell cycle control (p15INK4b), cell invasion and adherence (E-cadherin, H-cadherin, CTNNA1, Trombospondin), apoptosis (BCL2L10, DAPK1 and RIL), signal transduction (SOCS1, RASSF1A), hormonal response (Calcitonin and estrogen receptor), and the Wnt pathway (FZD9) (Table 1).[4-19] As a proof of principle, in therapy-related MDS, which can be considered as an in-vivo model for poison-induced leukemogenesis, hypermethylation of several genes was demonstrated.[6,7,10]

Table 1. Genes hypermethylated in MDS.

CELLULAR PATHWAY	*GENE*	*FREQUENCY OF METHYLATION (%)*	*REFERENCES*
DNA REPAIR	MGMT	2%	13
CELL CYCLE CONTROL	CDKN2B (p15)	23-80%	5, 6, 11, 15
CELL INVASION AND ADHERENCE	CDH1 (E-cadherin)	15-70%	11, 15, 16
	CTNNA1	10-31%	12, 14
	TSP1 (Trombospondin)	6%	16
APOPTOSIS	BCL2L10	12%	17
	DAP-kinase 1	15-47%	7, 16, 18
	RIL	36-70%	15
SIGNAL TRANSDUCTION	SOCS1	31-47%	8
	RASSF1A	9%	9
HORMONAL RESPONSE	Calcitonin	50%	4
	Estrogen receptor	7-19%	11, 15
WNT PATHWAY	*FZD9*	*>50%*	*19*
-	HIC1	31%	11

Later on, techniques for whole genome analysis of DNA methylation became available and it was shown that global hypermethylation is a marker of disease progression, associated to unfavourable prognosis. Hypermethylation in MDS characterizes genes located on all chromosomes, is more frequent than cytogenetic aberrations and cooperates with chromosomal deletions in determining loss of heterozygosity.19,20 In this line, the Wnt-pathway member FZD9 encoded on chromosome 7q resulted aberrantly methylated in many patients with chromosome 7q deletions, and predicted for unfavourable clinical outcome.19 The "hypermethylator phenotype" involving al least 10 genes from the DNA

repair, cell-cycle control, differentiation, apoptosis and detoxification pathways was shown to be relevant for leukemogenesis and predictive of poor prognosis, independent of IPSS.15

More recently, mutations or deletions of genes involved in DNA methylation (DNMT3A, IDH1, IDH2, and TET2) and in the regulation of histone function (ASXL1, EZH2, and UTX) have been detected in MDS[21-28] (Table 2). These mutations may be the genetic basis for the frequent epigenetic changes in MDS. Indeed, it has been shown that IDH1/IDH2 mutations are associated to histone demethylation and, together with TET2 mutations, to DNA hypermethylation.[29-31] The major functional role of epigenetic mutations is also shown by their significant prognostic impact in MDS.[32,33]

Table 2. Genetic mutations of epigenetic regulators in MDS.

Pathway	*GENE*	*FUNCTION*	*FREQUENCY OF MUTATIONS OR DELETIONS (%)*	*REFERENCES*
DNA METHYLATION	DNMT3A	DNA Methyltransferase	2-8%	25, 26, 28
	IDH1	2-hydroxyglutarate (2HG) production	3-4%	28, 32
	IDH2	2-hydroxyglutarate (2HG) production	11%	28
	TET2	Cytosine hydroxymethylation	19-25%	21, 22, 28
HISTONE FUNCTION	ASXL1	Polycomb-group protein (PCG)	10-16%	23, 24, 28
	EZH2	Histone methyltransferase (PCG)	2-6%	27, 28
	UTX	Histone methyltransferase (PCG)	Rare	23

The potential reversibility of epigenetic changes has made chromatin remodeling enzymes attractive targets for therapeutic intervention, opening up new horizons for the treatment of MDS. Two classes of epigenetic drugs inhibiting DNMTs and the HDACs enzymatic activities are available in clinical settings. DNMT inhibitors (DNMTi) have shown significant clinical activity in MDS.13,34-36 In particular the combination of DNMTi 5-azacytidine and the histone deacetylase inhibitor valproic acid was active and safe in patients with higher risk MDS enrolled in the GIMEMA MDS0205 multicenter trial. In particular the achievement of therapeutic levels of valproic acid (>50 microgram/microliter) might improve the response to azacytidine.35

Although therapeutic activity of hypomethylating agents has been observed in several studies, and the epigenetic mechanism of action is well-known, biologic predictors of response have not been identified yet. In this line, we have reported that methylation and expression changes induced by hypomethylating treatment are complex and several pathways are involved in treatment outcome.[17] Among these, hypermethylation of the apoptotic gene BCL2L10 was associated to a shorter survival.[17,38] Most recently, the discovery of the hypomethylating function of the TET2 enzyme, impaired in MDS due to mutations has given new hopes for the identification of epigenetic response predictors.[30,37-39]

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GENETIC PREDISPOSITION TO MYELOPROLIFERATIVE NEOPLASMS

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Myeloproliferative neoplasms (MPN) comprise a heterogeneous group of hematological disorders characterized by a clonal overproduction of differentiated myeloid cells, propensity to thrombosis, hemorrhage, and increased risk of leukemia. The 2008 WHO classification identifies seven distinct categories of Ph-negative MPN including essential thrombocythemia (ET), polycythemia vera (PV), primary myelofibrosis (PMF), mastocytosis, chronic eosinophilic leukemia not otherwise specified (CEL-NOS), chronic neutrophilic leukemia (CNL), and MPN unclassifiable (MPN-U).[1] An important breakthrough in the understanding of the so called "classic Ph-negative MPN", namely ET, PV, and PMF, was reached in 2005 with the discovery of the V617F point mutation of the *JAK2* gene. This mutation affects the pseudokinase domain (JH2, exon 14) of the protein. The mutant JH2 can no longer autoinhibit the kinase domain JH1, leading to constitutive activation of the kinase.[2-5] Beside the *JAK2* V617F mutation, present in about 60% of MPN cases, other less frequent somatic mutations have been associated with MPN, such as *MPL*, *TET2*, *CBL*, and *EZH2* mutations.[6]

A genetic predisposition to the acquisition of these various somatic mutations might be hypothesized. Some lines of evidence are consistent with this hypothesis:

1) Familial clustering of MPN

Although MPN have in most instances a sporadic occurrence, familial clustering of MPN have been described.[7,8] The prevalence of familial cases among apparently sporadic MPN is 7.6%.[9] Relatives of MPN patients have a 5- to 7-fold increased risk of developing MPN.[10] Common mutations involved in the pathogenesis of MPN are generally not inherited, but somatically acquired also in familial cases, with the exception of isolated observations. A germline TET2 mutation has been described in a PV patient,[11] while inherited CBL mutations have been shown to cause juvenile myelomonocytic leukemia.[12] Overall, the genes that frequently mutate somatically in MPN do not significantly contribute to familial MPN and in the majority of familial cases the causative germline mutation is still unknown.

2) Predisposing haplotypes

Independent studies have identified a particular *JAK2* gene haplotype (46/1 or GGCC) that predisposes to *JAK2* V617F positive MPN.[13-15] Subsequent studies revealed that also *JAK2*-ex12 mutations[16] and *MPL* mutations[17] are associated with the *JAK2* GGCC haplotype. There are two plausible hypotheses to explain this interaction between germline and somatic genetic variants. The hypermutability hypothesis postulates that the high risk haplotype could confer a hypermutability property on the *JAK2* locus; carriers of the risk haplotype would more frequently acquire mutations in *JAK2*.

The alternative hypothesis is that the *JAK2* V617F mutation occurs at the same rate on the different haplotypes, but cells in which it lands on the risk haplotype gain a stronger selective advantage.[18]

JAK2 GGCC haplotype does not explain familial clustering as the haplotype frequency do not differ between familial and sporadic cases.[19]

More recently a single nucleotide polymorphism (SNP) within the *JAK2* promoter region rs 1887428) has been associated with MPN. In patients carrying the high risk SNP there is an enhanced transcription factor binding at rs 1887428 that leads to increased JAK2 expression and confers a selective advantage on cells containing the risk haplotype. So this risk haplotype would contribute to MPN pathogenesis through allele-specific transcription factor binding and JAK2 expression.[20]

3) Germline *JAK2* mutations

Germline mutations underlying familial syndromes resembling MPN have been reported. In particular, germline mutations in thrombopoietin (*THPO*) and thrombopoietin receptor (*MPL*) gene have been shown to be responsible for hereditary thrombocytosis.[21-24] Familial MPN with thrombocytosis and hereditary thrombocytosis are traditionally considered two separated entities, with different pattern of proliferation (clonal multilineage involvement in the former, polyclonal proliferation of the megakaryocytic lineage in the latter) and different transmission modalities (complex in the first and autosomal dominant in the second). In hereditary thrombocytosis mutations of the thrombopoietin or *MPL* genes are disease causing defects, whilst *JAK2* mutations are usually considered a secondary event in familial MPN.[25] This last assumption should be reviewed according to recent observations. Two cases of hereditary thrombocytosis associated with novel *JAK2* germline mutations (R564Q and V617I) have been recently reported.[26,27]

In conclusion, all these observations provide a strong rationale for searching for predisposition genes to MPN using the latest genomics approaches (genome-wide detection of somatic lesions, SNP-array based linkage analysis, next generation sequencing). Novel diagnostic and therapeutic targets might emerge as result of these studies.

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INSIGHTS INTO THE MOLECULAR PATHOGENESIS OF PROGRESSION IN CHRONIC MYELOPROLIFERATIVE NEOPLASMS

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Philadelphia chromosome-negative myeloproliferative neoplasms (MPNs) are clonal myeloid disorders with a clinical course usually chronic in nature, but some patients show disease progression (to secondary myelofibrosis in case of previous polycythemia vera (PV) or essential thrombocythemia (ET) or to an accelerated phase in case of myelofibrosis (MF)) and/or leukemic transformation. The latter occurs in about 10-15% of MF patients and in 3-5% of PV and ET patients. In recent years, great progress has been made in the understanding of the molecular pathogenesis of MPNs. The *JAK2V617F* mutation has been detected in >95% of patients with PV and 60% of ET or primary MF patients. Additional mutations have been found in other genes including *MPLW515L/K*, *TET2, EZH2, ASXL1* among the others and in exon 12 of *JAK2*. All of these mutations can contribute to the molecular pathogenesis of MPNs. On the other hand, the genetic mechanisms of transformation are largely unknown, and the complement of genetic abnormalities in MPN patients who undergo LT remains a mistery. Mutations in *FLT3, NRAS, NPM1, RUNX1, DNMT3A, IDH1/2, TP53 and TET2* have all been implicated in the process of leukemic transformation, as well as chromosomal aberrations including deletions of *IKZF1, JARID2* and amplification of *MDM4*, a TP53 inhibitor. At least in some cases, leukemia can arise on the background of the clone associated with the development of the chronic phase or arise independently from it, as suggested by the lack of *JAK2V617F* mutation in the leukemic balsts in some cases, particularly from a previous MF, at the time of leukemic transformation. Secondary leukemias arising from a previous MPN (sAML) are prognostically very dismal and have very short survival, pointing to substantial differences compared to the de-novo leukemias. In a recent study by Milosevic et al (2012) high-resolution genotyping and loss of heterozigosity (LOH/UPD)) analysis were performed in 86 patients with sAML, allowing to define that mutations in *TP53,* 9pUPD and del7q (where CUX1 maps) were significantly associated with sAML opposite to NPM1 and FLT3 mutations typical of de-novo AML. Multivariate analysis identified mutant *TP53* as a potent independent adverse prognostic factor in sAML. Of interest, other mutations such as *IDH1/2*, *CBL* and *NRAS* did not distinguish between secondary and de-novo AML. Deletions of the short arm of chr6 are aso quite frequent in sAML, containing a commonly deleted region of about 1.1Mb that contains *JARID2*, a member of the polycomb repressive complex 2 (PRC2); del6p were strongly associated with the leukemic phase of MPNs. Other members of the PRC2 have been found mutated in sAML, including *AEBP2, SUZ12 and EZH2*, suggesting an essential role of PRC2 in the leukemic transformation. Over 21% of patients with blast phase-related MPN carry an IDH1/2 mutation, and this was irrespective of *JAK2V617F* status. This appears to indicate that IDH1/2 mutations can also influence the transformation of MPN to blast phase disease. Moreover, *LNK, CBL* and *RUNX1* mutations were also found in leukemic blasts of patients implying that *these* mutations may represent one of the most frequent genetic events implicated in MPN transformation to AML.

Therefore there is growing evidence that multiple mutational events contribute to the pathogenesis of leukemia after MPN as the result of clonal evolution expressing a high degree of genetic instability. It is very likely that new analytical techniques including next generation sequencing approach could help to novel mutations and at the same time establish a common pathogenetic theme in the secondary leukemias after MPN.

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EHA ACTIVITIES FOR YOUNG HEMATOLOGISTS

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Introduction. One of EHA's missions is to promote and support high quality research by providing funding to promising junior clinical, basic and experimental researchers in the field of malignant and non-malignant hematological diseases. Various programs have been established to encourage young hematologists in their development to becoming an excellent researcher and expert in their field. Since 2000, a number of different fellowships have been established by EHA or jointly with partner societies.

In addition to the fellowship program, the EHA-ASH Translational Research Training in Hematology (TRTH) was established in 2010 and provides junior researchers with a unique, year-long training and mentoring experience. This global program is focused on helping early-career scientists build successful careers in hematologic translational research.

EHA Fellowships program. Each fellowship offers a grant fund to conduct the awarded research project in the home or host institute for a defined period of time. The EHA research and José Carreras Young Investigator fellowships provide financial support for talented young researchers and mobility within and beyond Europe is positively assessed. The partner fellowships are dedicated to junior hematologists from new accession and European Union candidate countries and developing countries to facilitate opportunities to work at Western European institutes. The EHA-ISTH*) Fellowship is intended to support the study of physiology of coagulation, bleeding or thrombosis and the EHA-JSH**) Exchange program is intended to provide European and Japanese research institutes the opportunity to exchange scientists reciprocally for a short period. Focus of the last mentioned program is on hematological disorders, differences between Europe and Japan, determining factors and exchanging of new information and technologies.

All applications are reviewed by a panel of experts and selection is based on originality, clarity and feasibility of the research proposal, the CV of the applicant and the institute. So far, over 70 hematologists have been awarded and the development of their career is monitored by the EHA continuously. Some former awardees are involved in other EHA activities or positions in EHA committees and their contribution is highly valued.

EHA-ASH Translational research training in hematology. The goal of the TRTH award is to give researchers the tools, mentoring and access to resources necessary and beneficial for a successful career in translational research in hematology. Twenty early-career scientists are selected each year to participate in this rigorous training program. So far, 60 trainees have participated in the program. TRTH faculty is made up of international leaders in hematology who cover e.g. pathogenesis, diagnostics, and experimental treatment of hematological disorders and will focus on specific scientific methodology adapted to the needs of the participants, as well as on more general subjects with relevance for planning and pursuing a translational research project.

The TRTH program contains a week-long spring course including didactic and working group sessions, a meeting at the EHA Annual Congress where trainees work on continuing refining their research proposals, and a meeting at the ASH Annual Meeting where trainees make a final presentation on the status of their research. Attendance at all three meetings is mandatory. The program offers a unique opportunity to network and interact with key leaders in hematology in Europe and the USA and to build on global collaborations for the future.

Aims and Policy. With the Career Development program EHA supports excellent research in hematology and its subspecialties and encourages the future of this medical discipline. Hematological research is invaluable for the diagnosis and treatment of benign and non benign hematological disorders. Furthermore, hematological research is crucial in the diagnosis of various non-hematological diseases and plays a vital role in approaching aging problems.

The promotion of the career development of young talented researchers in this field will contribute to the evolvement of the field and will indirectly be an impetus for the society.

For more information on the eligibility criteria and application to the EHA's Career Development program visit www.ehaweb.org.

*) ISTH: International Society of Thrombosis and Hemostasis.
**) JSH: Japanese Society of Hematology.

NEW INSIGHTS IN THE MOLECULAR BASIS OF MYELODYSPLASTIC SYNDROMES

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Chromosomal abnormalities are detected in approximately 50% of patients with myelodysplastic syndrome (MDS) by conventional cytogenetic analysis. Deletions of chromosome 5q are the most frequent single chromosomal abnormality in MDS. Recently, functional studies have implicated the haploinsufficiency of *RPS14*, a component of the 40S ribosomal subunit, in the pathophysiology of 5q- syndrome[1]. Depletion of ribosomal proteins results in TP53 overexpression leading to cell cycle arrest in erythroid progenitor cells.[2] In addition, decreased expression of two micro-RNAs in the common deleted region, *miR-145* and *miR-146a*, are implicated in dysregulated megakaryopoiesis in this disorder.[3]

Acquired somatic mutations in several genes have been recently identified in MDS. The mutated genes can be classified according to the function of the proteins they encode: (i) splicing factors (*SF3B1, U2AF1, ZRSR2, SRSF2, SF3A1, PRPF40B, U2AF2,* and *SF1*), (ii) epigenetic regulators (*TET2, DNMT3A, IDH-1* and *-2*, *ASXL1, ATRX,* and *EZH2*), (iii) transcription factors (*RUNX1* and *ETV6*), (iv) kinase signalling (*JAK2, NRAS/KRAS, PTPN11, MPL*, and *CBL/CBLB*), (v) DNA damage pathway (*TP53*).

Mutations of *SF3B1* were found in 25-30% of MDS patients, but the proportion of positive patients was significantly higher in sideroblastic categories (RARS and RCMD-RS) (70%).[4,5] Interestingly, a high prevalence of *SF3B1* mutations was found in patients with equal or more than 15%, as well as in those with less than 15%, bone marrow ring sideroblasts. The association between *SF3B1* mutation and ring sideroblasts was confirmed across all the myeloid neoplasms, including myeloproliferative neoplasms (MPN) and myelodysplastic/myeloproliferative disorders (MDS/MPN). In fact, among MDS/MPN a high prevalence of *SF3B1* mutations was also observed in patients with RARS associated with marked thrombocytosis (RARS-T).[6] In addition, somatic mutations of *SF3B1* were also observed in a small fraction of patients with primary myelofibrosis, and in all cases sideroblasts were detected in the bone marrow.[7] *SF3B1* mutations were associated with down-regulation of key gene networks, including core mitochondrial pathways.[4] Mutations of *SF3B1* were found to be independent predictors of favourable clinical outcome.[6]

Aberrant DNA methylation has been implicated in the pathogenesis of MDS. *TET2* mutations have been identified in approximately 25% of patients with MDS,[8] and cause a loss of function of the TET2 enzyme that converts 5-methylcytosine to 5-hydroxymethylcytosine in a reaction dependent on a-ketogluterate, which is produced by *IDH1* and *IDH2*.[9] Interestingly, mutations in *IDH1/2* and *TET2* are mutually exclusive in acute myeloid leukemia and are associated with similar epigenetic profiles, implying a common effect on epigenetic regulation. Mutations in *DNMT3A* were identified in 8% of MDS patients. Experiments in mice showed that *DNMT3A* null hematopoietic stem cells had increased self-renewal capacity and impaired differentiation, but mice did not develop hematologic malignancies, suggesting that transformation requires cooperating mutations.[10]

The application of next generation sequencing analysis will allow in the near future to identify the complete spectrum of somatic genetic alterations that cause MDS. This will allow to identify molecular markers that will substantially improve our ability to diagnose MDS, to establish specific correlations between genotypes and disease phenotypes, to accurately estimate individual patient risk and predict response to therapy.

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CLINICAL RELEVANCE OF HLA-DPB1 DISPARITY AFTER UNRELATED STEM CELL TRANSPLANTATION

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HLA-DP antigens are heterodimers encoded by the DPA1 and the DPB1 locus, which are in close linkage disequilibrium (LD) between each other and display limited and high allelic polymorphism, respectively. 176 DPB1 alleles have been described to date (http://www.ebi.ac.uk/imgt/hla), which arose mostly by shuffling of six hypervariable regions encoded within the polymorphic exon 2. In allogeneic hematopoietic stem cell transplantation (HSCT) from unrelated donors (UD) matched for HLA-A,B,C,DRB1 and DQB1 (10/10 matched UD), DPB1 is mismatched in over 80% of the cases, due to low LD between DPB1 and the other two HLA class II beta chain loci. Donor T cell alloreactivity to mismatched HLA-DP antigens expressed by the patient's residual leukemia has been shown to mediate graft versus leukemia activity after UD-HSCT, resulting in significantly lower risks of leukemia relapse after DPB1 allele mismatched compared to DPB1 allele matched transplantation (1,2). On the basis of alloreactive T cell cross-reactivity patterns, DPB1 alleles can be classified into at least three distinct T cell epitope (TCE) groups (3-5). Donor-recipient DPB1 mismatches involving alleles from different TCE groups (DPB1 TCE non-permissive mismatches) were shown to be associated with significantly higher risks of adverse clinical outcome in terms of overall and non-relapse mortality as well as acute graft versus host disease, compared to those involving alleles from the same TCE group (DPB1 TCE permissive mismatches), after HSCT from UD matched for 10/10 or 9/10 of the non-HLA-DPB1 alleles (3, 5-6). In particular, the probability of survival was similar after 10/10 matched transplantation from DPB1 TCE non-permissive UD, compared to 9/10 matched transplantation regardless of DPB1 TCE matching status. This was shown by retrospective analysis of 8539 UD-HSCT facilitated by the International Histocompatibility Working Group in Hematopoietic Cell Transplantation (6). Based on these data, we conclude that prospective donor-recipient DPB1 typing and avoidance of DPB1 TCE non-permissive mismatches can significantly reduce the clinical risks associated with UD-HSCT. A webtool for assessment of the DPB1 TCE matching status on the basis of donor-recipient DPB1 allele typing will shortly be available at http://www.ebi.ac.uk/imgt/hla.

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IMPACT OF POLYMORPHISMS OF GLUTATHIONE HOMEOSTASIS ON THE OUTCOME OF PATIENTS RECEIVING AN ALLOTRANSPLANT CONDITIONED WITH BUSULFAN

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Allogeneic hematopoietic stem cell transplantation (HSCT) is the only curative option for many hematologic diseases. Transplant related mortality (TRM) allogeneic HSCT depends mainly on graft-versus host disease (GVHD), infections and toxicity from the preparative regimen. HSCT is a systemic stress and its outcome can be considered as the successful functioning of multiple stress response systems, and it is therefore expected to be modulated by the inter-individual variability at a large array of genetic loci *(Chien, et al., 2012)*. Polymorphisms in non-HLA loci have also been associated with HSCT outcome *(Chien, et al., 2012; Mullally et al., 2007)*. Major attention has been focused to the relationship between genetic variation at innate immunity and inflammatory mediators with GVHD *(Hansen et al., 2008)*. However contrasting results have been generated until now because of patents heterogeneity and small series analysed.

Busulfan, a bi-functional alchilating agent, is one of the most widely used drug in allogeneic HSCT conditioning regimens, in association with cyclophosphamide *(Santos et al., 1983)* and more recently with fludarabine *(Ciurea et al., 2009)*. Busulfan systemic exposure is an important parameter to monitor therapeutic efficacy *(Slattery et al., 1997; Gaziev et al., 2010)*. We analyzed 40 polymorphisms at 27 loci involved in hepatic glutathione balance *(Lu et al., 2009)*, in 185 patients who underwent allogeneic related or unrelated HSCT from 2005 to 2009, after a preparative regimen containing busulfan (busulfan cohort). The impact of such loci was tested on TRM, overall survival, and busulfan plasma levels. All the calculations were corrected for N=40 multiple comparisons. Censored data were analysed univariately according Kaplan-Meyer method and multivariately according to Cox model. GLM for repeated measures was used, as appropriate. In all analyses, genetic variables were implemented with the most important clinical variables, such as type of donor, age, intensity of conditioning, sex mismatch, interval diagnosis-transplant. The pharmacokinetics was available for 64 patients. Busulfan plasma concentration was determined by high-performance liquid chromatography HPLC before administration and at 15, 60, 120, 180, 240 minutes after dose 1 (day 1) and dose 9 (day 3).

We found that serine allele homozygosity at GSTA2 S112T locus is an independent prognostic factor for increased TRM, poorer survival and higher mean busulfan area under the concentration-curve, AUC). Intriguingly, the polymorphism is not predictive of clinical outcome for 146 concomitant transplants not receiving busulfan.

These data indicate that genetic inter-individual variability at GSTA2 is a prognostic factor for allotransplants receiving busulfan.

CD26, ANTI CD26 AND GvHD

Dominietto A

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CD26 or dipeptidyl peptidase IV (DPPIV) is a type II transmembrane glycoprotein expressed on the surface of epithelial, endothelial and lymphoid cells, particularly on CD4+CD45RO+ memory T cells, ubiquitous and with two main properties: the peptidase function and the binding activities to proteins both of host cells and microbes or viruses. DPPIV cleaves the N-terminal dipeptides from polypeptides containing alananine or proline in the penultimate position and regulates the activity of hormones, neuropeptides, chemokines such as RANTES (regulated on activation, normal T cell expressed and secreted), MDC (monocyte-derived chemokine), SDF-1 and SDF-1 (stromal cell-derived factors). Near the catalytic site the extracellular domain of the CD26 contains the adenosine deaminase (ADA) and the fibronectin binding residues. When CD26 binds to ADA, the enzyme converts adenosine irreversibly to inosine and reduces the extracellular concentration of adenosine that is a potent inhibitor of the T-cell activation. The complex DPPIV-ADA together with the binding of CD26 to others molecules such as tyrosine phosphatase CD45, CARMA-1 or caveolin-1, are important costimulatory factors leading to T cell proliferation and IL-2 production. DPPIV is also a receptor for fibronectin and collagen I suggesting a role in the adhesion of the T cells to the extracellular mathrix and in the regulation of the intracellular proteins trafficking.

As a result of its multifunctional activity CD26/DPPIV plays a role in the glucose homeostasis, is highly expressed in autoimmune diseases such as rheumatoid arthritis (RA) and multiple sclerosis (MS), is involved in the T-cell activation, interacts with the chemokine receptor CXCR4 and with the human immunodeficiency virus-1 transcription transactivator protein (HIV-1 Tat). Particularly in HIV infection DPPIV is involved with two different mechanism of action: the interaction with HIV-1 Tat inhibits the enzymatic activity of CD26 while the HIV-1 gp120 protein blocks the DPPIV-ADA interaction, leading to reduced T-cell activation.

The use of DPPIV inhibitors in animal models of human diseases such as diabetes type II, autoimmune disorders or transplantation shows a down-regulation of the immune system: inhibition of CD26 preserves pancreatic islet transplants through a pathway involving modulation of splenic CD4(+) T-cell migration in mice, prevents lung graft rejection in rats and suppresses autoimmune encephalomyelitis (EAE) in mice.

When translated into the clinical settings, the use of DDPIV inhibitors in type II diabetes reveals a benefit on glucose metabolism control, but is associated to an increased risk of infection complications, suggesting the potent role of CD26 inhibitors as immunosuppressive agents. Despite the observation that high levels of CD26+ T cells are detected in biological fluids or blood of patients with autoimmune disorders (MS, Graves's disease, RA), the use of DPPIV inhibitors into the clinical setting as target therapy remains to be determined.

In the allogeneic hematopoietic stem cell transplantation (HSCT) setting the intravenous monoclonal antibody anti CD26, named BT5/9, was used for the first time at our institution in 1985 for the treatment of steroids refractory acute graft versus host disease (GvHD). Four of eight patients received the BT5/9 showed a complete response, 2 a partial response and four patients are alive up to now. The antibody was thereafter no longer available for clinical use, and therefore was no longer used for the treatment of acute GvHD.

More recently, the murine monoclonal antibody against CD26 (BEGEDINA ®) has been isolated immunizing Balb/C mice with human activated t-lymphocytes. In the first pilot study, designed at our institution, 14 patients with steroids refractory acute GvHD was enrolled between December 2010 and February 2012. BEGEDINA ® was administered at the dosage of 2 mg/day for 5 consecutive days. The protocol was designed and proved safety and feasibility, with encouraging response rates. We have now opened a dose finding phase 2 clinical study: the primary end point is the interaction of BEGEDINA ® with at least 80% of circulating CD26+ cells in vivo, in patients with steroid resistant acute GvHD after allogeneic HSCT, with the aim of delivering an effective dose to modulated CD26+ cells.

PHARMACOGENETICS OF OLD AND NEW ANTICOAGULANTS

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Anticoagulants are among the most widely prescribed drugs in medicine. Warfarin is commonly used as life-long therapy in the prevention of systemic embolism, in patients with atrial fibrillation, valvular heart disease, and in the primary and secondary prevention of venous thromboembolism. Clinical management is difficult because of a narrow therapeutic range and a large inter- and intraindividual variability in treatment responses, rendering the clinical optimization of warfarin doses difficult. The dose requirements for warfarin have been shown to be influenced by various factors including age, weight, genetic background, vitamin-K enriched diet, drug interactions. Current clinical practice utilizes the international normalization ratio (INR) to optimize the dose of warfarin in individual patients which has performed far from ideal. Approximately 10% of Europeans require an unusually low dose of warfarin (1.5 mg/day or less) and these patients could be at increased risk of developing serious bleeds and undesirably high levels of anticoagulation, especially during the initial weeks of treatment. Recently, reference to genetic factors affecting response (both CYP2C9 and VKORC1) has been included in the prescribing information for warfarin in the USA by the Food and Drug Administration (FDA)

Current oral coumarin anticoagulants, such as warfarin, acenocoumarol and phenprocoumon, are likely to be replaced eventually by new oral anticoagulant drugs, which offer fixed dosing, more predictable pharmacokinetics and fewer interactions with drugs and food. The new drugs differ each other with respect to their mechanism of action and pharmacokinetics, especially with respect to elimination through the liver and kidneys. These drugs may potentially cause bleeding complications in patients with reduced drug excretion due to impaired hepatic and/or renal function. Genetic variation influences the absorption and efflux of drugs in the intestine, the metabolism of drugs in the liver and kidney and the effects of these drugs on their target proteins. Thus, it is conceivable that individual responses to drugs, both in terms of therapeutic effect as well as adverse effects, such as the risk of drug accumulation and bleeding may be modulated by several inherited differences.

FIBRINOLYTIC DISEASES: DO THEY REALLY EXIST?

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Diseases caused by alterations of specific factors of the fibrinolytic system are rare and often ill defined. Bleeding arising from the systemic intravascular activation of plasminogen may be encountered in hypotension, surgical trauma, heatstroke, prostate cancer, acute promyelocytic leukemia, and liver transplantation. The hallmark of these conditions is a striking increase of the circulating levels of plasminogen activators, resulting in the appearance of unchecked plasmin which, in turn, degrades numerous plasma proteins, including fibrinogen and some clotting factors. Often, however, systemic hyperfibrinolysis is a secondary response to widespread fibrin formation as in disseminated intravascular coagulation. Bleeding may also arise from the deficiency of fibrinolytic inhibitors. The best characterized is the rare deficit of plasmin inhibitor (α2-PI) which does not lead to a true hyperfibrinolytic state (i.e. plasminemia) but rather to an accelerated dissolution of fibrin (premature lysis). On the opposite side, fibrinolytic alterations that impair the removal of fibrin may favor venous and arterial thrombosis. Understandably, because thrombosis is a multifactorial disease, such fibrinolytic alterations might at best be viewed as predisposing conditions. The increase in plasminogen activator inhibitor 1 (PAI-1) is by far the most frequent finding in many conditions and diseases characterized by a heightened thrombotic risk. However, at variance with the prothrombotic alterations involving blood coagulation factors and inhibitors, neither PAI-1 nor any other fibrinolytic assay has gained a place in the thrombophilia screening panel. During the last two decades it has become increasingly clear that the fibrinolytic process may be altered even in the presence of an intact and totally normal fibrinolytic

system. This apparent paradox arises from the fact that several factors, traditionally unrelated to fibrinolysis, may affect the process of fibrin dissolution. Among the latter the most important is thrombin, which is able to slow down or even temporarily halt fibrinolysis through different mechanisms. A fibrin clot generated in the presence of a high thrombin concentration is resistant to plasmin-mediated degradation because of the formation of thinner and more packed fibrin fibers. Moreover, the huge amount of thrombin generated after the fibrin clot has already formed has a great impact on fibrinolysis for it activates factor XIII and TAFI (thrombin activatable fibrinolysis inhibitor), two strong clot stabilizers. Such a functional connection between blood coagulation and fibrinolysis suggests that derangements in thrombin formation, resulting from coagulation disorders or even platelet diseases, are likely to affect also the fibrinolytic process. Evidence from such a knock-on effect has been obtained in hemophilic patients who were shown to generate plasma clots that were much more susceptible to lysis than normal clots, a finding compatible with the fact that bleeding in hemophilic patients frequently occurs several hours or even days after injury (likely because of untimely removal of the hemostatic plug) and that, at least in certain circumstances, it can be prevented by antifibrinolytic agents. Moreover, in vitro studies suggest that thrombocytopenia makes blood clots more lysable largely because the reduction of the platelet procoagulant surface leads to less thrombin generation. Finally, experiments in patients with a hypercoagulable state (e.g. carriers of the prothrombin mutation or FV Leiden) indicate that their clots are more resistant to fibrinolysis due to the heightened thrombin formation. Therefore, considering the wide spectrum of diseases affecting directly or indirectly the clotting system, it can be hypothesized that the alterations of the fibrinolytic process are actually much more frequent than previously thought. From the clinical standpoint this implies that the use of drugs interfering with the fibrinolytic process (either inhibitors or stimulators) might represent a new therapeutic approach in many thrombotic and bleeding disorders beyond those classically scored as fibrinolytic diseases.

NEW HORIZONS IN THE DIAGNOSIS AND TREATMENT OF LYMPHOID MALIGNANCIES

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B cell non-Hodgkin lymphoma (B-NHL) comprises a heterogeneous group of neoplasms derived from the malignant transformation of mature B cells at various stages of differentiation.[1] In most lymphoma types, the final clonal expansion involves antigen-stimulated B cells that have experienced the germinal center reaction, a unique microenvironment where lymphocytes undergo intense proliferation and remodeling of their immunoglobulin genes by the process of somatic hypermutation (SHM) and class switch recombination.[2] Indeed, errors occurring during these DNA remodeling events are responsible for two major mechanisms of genetic lesion associated with these malignancies, namely chromosomal translocations and aberrant somatic hypermutation.[3,4] Among B-NHL, diffuse large B cell lymphoma (DLBCL) represents a particularly compelling problem, since this aggressive form accounts for 30-40% of all diagnoses and, despite the development of treatment regimens that have greatly extended the life expectancies of DLBCL patients, nearly 50% of cases are not cured by available therapeutic approaches. Such lack of success is partly due to the marked phenotypic, molecular and clinical heterogeneity of this malignancy,[1] as also recognized by gene expression profile analyses.[5] Consistent with this heterogeneity, a multitude of genetic alterations have been identified as recurrently assoc ated with DLBCL, many of which segregate with individual phenotypic subtypes, i.e. the germinal center B cell-like (GCB) DLBCL and the less curable activated B celllike (ABC) DLBCL, suggesting the involvement of distinct oncogenic pathways[6-11] However, the lesions that had been discovered so far likely represent only a fraction of those that are necessary for malignant transformation. Thus, the identification of additional critical pathways that are involved in tumor initiation and maintenance remains a major need towards an improved understanding of these malignancies and the development of more effective, rationally targeted therapies.

In recent years, the availability of next-generation sequencing technologies combined with genome-wide copy number analysis has revolutionized the field by allowing to define the full spectrum of genetic lesions that are present in the DLBCL genome in a comprehensive and unbiased manner. These studies revealed the involvement of multiple biological pathways that are dysregulated by genetic lesions in a large fraction of DLBCL patients and had not been previously implicated in this malignancy. Among the newly identified candidates, genes encoding for histone modification enzymes emerged as the most common targets of structural alterations in this disease[12-15] . Nearly 30% of DLBCLs harbor somatic mutations and/or deletions inactivating CREBBP and, less frequently, EP300, two histone and non-histone acetyltransferases involved in multiple cellular functions, including the negative regulation of the BCL6 proto-oncogene activity and the activation of the p53 tumor suppressor[12-14]. Moreover, as many as 40% of DLBCL cases, independent of their phenotypic subtype, carry monoallelic or biallelic mutations that disrupt the *MLL2* mehtyltransferase[13-15]. Importantly, these same genes are also recurrently mutated in a large proportion of follicular lymphomas[12,14], the second commonest lymphoma type (40% and 89%, respectively), indicating a central role for epigenetic regulators of chromatin structure in the pathogenesis of B-NHL. In addition to histone/chromatin modification genes, multiple truncating mutations were identified in genes that are required for immune recognition and surveillance, such as *B2M* and *CD58*, suggesting that their loss may contribute to malignant transformation by favoring evasion of the tumor cells from the immune system[16]. Finally, approximately 11% of DLBCL cases carry activating mutations in the MEF2B transcription factor[13-15], which serves as a master regulator of germinal center responses, in part by modulating the activity of BCL6.

The identification of these lesions has relevant clinical implications for both the diagnosis and treatment of DLBCL, as it provides the basis for the development of rational therapeutic approaches that may target specific pathways in distinct DLBCL patients.

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NEW HIGH-THROUGHPUT ANALYSIS OF DNA FOR 2020 PROGRAMS

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DNA sequencing technology has revolutionized biology and driven a

massive acceleration in research and development. The chain termination methodology developed by Fredrick Sanger in the 1970's has, until recently, been the most widely used sequencing method. The demand for genome sequence data using the Sanger methodology drove the formation of sequencing centers and collaboration on a global scale but, due to the scale of the task and the cost, only a limited number of species were covered. Since the late 1990's, researchers in both academia and industry have made efforts to develop alternative approaches for DNA sequence determination, looking to obtain greater sequencing throughput and a cost effective sequencing technology.

The commercial launch of the first massively parallel pyrosequencing platform in 2005 opened the new era of high-throughput genomic analysis, now referred to as next-generation sequencing (NGS). As a massively parallel process, NGS generates hundreds of megabases to hundreds of gigabases of nucleotide sequence output in a single instrument run, depending on the platform. The major commercially available NGS platforms in this new market include Illumina, Roche, Life Technologies, Helicos and Pacific Biosciences. In general, these technologies offer faster and much cheaper (cost-per base / cost-per-read) sequencing than the existing Sanger methodology, but with a shorter read length or higher error rate.

NGS machines are democratizing high throughput sequence generation, enabling investigators to conduct experiments that were previously not technically feasible or affordable. On the one hand this creates great opportunity, sequencing more genomes more quickly, opening up new lines of research and revitalizing others, and potentially deploying sequencing in new technological contexts. On the other hand, the unprecedented volumes of data generate by NGS experiments are posing challenges in terms of data transfer, storage and computational analysis that as a side effect is also leading to advances in bioinformatics and information technology applied to the solution of biological problems.

Nevertheless, NGS technologies are having a striking impact on genomic research and the entire biological field. With its ability to tackle the unsolved challenges, NGS is unraveling the complexity of the human genome in terms of genetic variations, some of which are being confined to susceptible loci for some common human conditions. By documenting the contributions of the human genomic variation in medicine, NGS technologies shiftg the way the medical profession views many diseases

BEST ABSTRACTS

BEST-01

DIAGNOSTIC AND PROGNOSTIC IMPLICATIONS OF NOTCH2 MUTATIONS IN SPLENIC MARGINAL ZONE LYMPHOMA

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Introduction. By performing whole exome sequencing analysis of splenic marginal zone lymphoma (SMZL), our group has recently observed that mutations of NOTCH2, a gene required for marginal-zone development, are the most frequent genetic lesion of this lymphoma type. This study aimed at assessing the diagnostic and prognostic relevance of NOTCH2 mutations in SMZL. *Methods*. NOTCH2 mutations were investigated by targeted Sanger resequencing across the clinico-pathologic spectrum of mature B-cell tumors (n=516). Survival was analysed by the Kaplan-Meier method and compared by the log-rank test. *Results*. NOTCH2 mutations occurred in 25/117 (21.3%) SMZLs, and were represented in all instances by truncating events (14 frameshift indels and 11 nonsense mutations), that clustered within a hotspot region in exon 34, including a recurrent p.R2400* nonsense mutation in 6/25 (24.0%) cases (Figure 1A). NOTCH2 mutations were consistently absent in the heterodimerization domain or in other portions of the gene that are known to be targeted by inactivating mutations in different cancer types. Based on their distribution, all NOTCH2 mutations were predicted to cause impaired degradation of the NOTCH2 protein through the elimination or truncation of the C-terminal PEST domain, which is required for proteasomal recruitment and degradation of NOTCH2. Analysis of paired normal DNA confirmed the somatic origin of the mutations in all cases for which germline material was available (n=13). In 3 patients for which multiple samples were available from different involved organs, the same NOTCH2 mutation was detectable at all sites, namely peripheral blood, bone marrow and spleen, suggesting that the mutation had been acquired before dissemination of the lymphoma clone. NOTCH2 mutations were consistently absent in nodal marginal zone lymphoma (0/18), chronic lymphocytic leukemia (0/100), mantle cell lymphoma (0/20), follicular lymphoma (0/20), hairy cell leukemia (0/20) and multiple myeloma (0/22), and occurred sporadically in extranodal marginal zone lymphoma (1/65, 1.5%), and diffuse large B-cell lymphoma (5/134, 3.7%) (Figure 1A). The clinical impact of NOTCH2 mutations on SMZL overall survival (OS) was assessed in 94 patients with available follow-up. At 5 years, SMZL patients harboring NOTCH2 mutations were characterized by a significantly higher OS probability (93.3%; 95% CI: 80.0-100%) compared to patients harboring a wild type NOTCH2 (74.3%; 95% CI: 61.4-87.2%; P=.048) (Figure 1B). *Discussion*. These data document that, among B-cell malignancies, NOTCH2 activating mutations are predominantly associated with SMZL, and underscores the genetic individuality of SMZL versus other marginal zone-derived lymphomas and versus indolent B-cell lymphoproliferative disorders clinically mimicking SMZL. Also, the results of this study provide a rationale for the design of novel therapeutic strategies for SMZL targeting the NOTCH signaling.

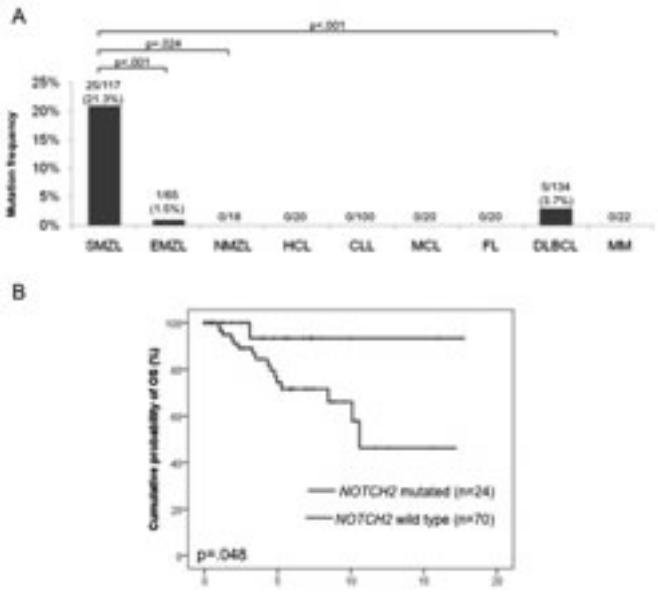

Figure 1.

BEST-02

CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) WITH STEREOTYPED IGHV GENES BELONGING TO SUBSET #1 EXHIBITS DISTINCTIVE GENETIC, GENOMIC AND FUNCTIONAL FEATURES

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Introduction. Chronic lymphocytic leukemia (CLL) cases with stereotyped B-cell receptor (BCR) belonging to subset #1 (IGHV1-5-7/IGKV1-39) display a poor outcome. We aimed to characterize the genetic and genomic features, as well as the BCR function of this subgroup of patients. *Methods.* From a series of 579 CLL patients, we selected 20 subset #1 CLL and evaluated the prognostic markers (CD38, ZAP70, FISH lesions, TP53 mutations), prognosis, gene expression profile and *in vitro* response to IgM stimulation. Prognosis was evaluated as treatment-free interval (TFI), calculated from the time of diagnosis to the first treatment, death or last follow-up. For microarray analysis, HGU133 Plus 2.0 Affymetrix arrays were used. Proliferation assay was measured by [3H]thymidine uptake on CLL cells, after 24 and 48 hours of stimulation with a F(ab')2 anti-human IgM. *Results.* Subset #1 CLLs displayed unmutated IGHV genes in all cases and were highly associated with the presence of del(11q) (50%) in comparison with unmutated (P=0.054) and mutated CLL (p <0.0001), and with cases utilizing the same IGHV genes but with a heterogeneous HCDR3 (non-subset #1 CLL) (P=0.01); there were no distinctive features regarding gender distribution, CD38, ZAP70, CD49d expression, lymphocyte morphology and TP53 mutations. TFI of subset #1 patients was significantly shorter in comparison with the whole cohort of unmutated and mutated CLL (Log-rank test, p <0.0001), non-stereotyped unmutated and mutated CLL (p <0.0001) and non-subset #1 CLL (P=0.02). Microarray unsupervised analysis on 80 unmutated/mutated/stereotyped/non-stereotyped CLL showed a significant enrichment of subset #1 cases in the same cluster, most likely associated to the presence of stereotypy rather than the IGHV mutational status, suggesting a common transcriptional signature for this subgroup. A supervised approach comparing subset #1 vs. non-subset #1 cases corroborated this finding and identified several differentially expressed transcripts involved in BCR signal transduction (LYN, BTK, PTPN6, NPM1), apoptosis regulation (ATM, PARP1, BIRC3), cell proliferation (KRAS, RASD1) and oxidative processes (CREB1, CREB3L2), regardless of del(11q). Accordingly, *in vitro* BCR ligation with anti-IgM revealed a significantly higher proliferation of subset #1 vs. non-subset #1 CLL (all unmutated), both at baseline (P=0.0098) and after 24 (P=0.0035) and 48 hours (P=0.009) upon stimulus. *Conclusions.* Subset #1 CLL show distinctive genetic and genomic features, as well as *in vitro* proliferative response, representing a paradigmatic example of the direct link between BCR structure, function and patients prognosis. Our findings also suggest that subset #1 CLL may represent an ideal candidate for treatment with inhibitors of the BCR pathway, recently utilized in clinical trials.

BEST-03

EITHER ALLOGENEIC OR AUTOLOGOUS BCR-ABL-SPECIFIC CTL INFUSIONS ARE ASSOCIATED WITH DURABLE MRD CLEARANCE AND RECOVERY OF ANTI-LEUKEMIC T CELLS IN PH+ALL PATIENTS ON IMATINIB

Riva G, Comoli P,[1] Barozzi P, Basso S,[1] Lagreca I, Quadrelli C, Quartuccio G,[1] Vallerini D, Zanetti E, Forghieri F, Paolini A, Fantuzzi V, Coluccio V, Bresciani P, Cuoghi A, Morselli M, Maccaferri M, Pioli V, Marasca R, Narni F, Zecca M,[1] Luppi M, Potenza L

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Our previous studies showed that autologous BCR-ABL-specific cytotoxic T lymphocytes (CTLs) may be often detected in the bone marrow (BM) of Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph+ALL) patients upon tyrosine kinase inhibitors (TKIs), and that their frequencies inversely correlated with minimal residual disease (MRD) values (Riva *et al*., Blood 2010). These data suggest that BCR-ABL-specific CTLs may be involved in the control of Ph+ALL, providing the rationale to develop BCR-ABL-targeted adoptive cell treatment.

In this study, BCR-ABL-specific CTLs were successfully generated from long-term *in vitro* cultures of peripheral blood mononuclear cells (PBMCs) in 5/6 patients, using, as leukemia-specific antigen stimulation, 9-20mer peptide pools derived from p190BCR-ABL protein fusion region. Pre-clinical functional analyses of such CTL lines showed highly-specific IFNg production and lytic activity toward leukemic targets, with limited alloreactivity.

Two Ph+ALL patients, pt.1 and pt.2, the former showing Ph+ALL molecular relapse after allogeneic HSCT and persisting MRD despite donor lymphocyte infusion and imatinib treatment, the latter with persistently high MRD levels albeit prolonged high-dose imatinib therapy, were compassionately treated with monthly incremental doses of BCR-ABL-specific CTLs (from $1x10^5$/Kg to $5x10^5$/Kg in pt.1; from 1x10^6/Kg to $3x10^6$/Kg in pt.2), allogeneic in the first case, autologous in the second. None of the patients experienced any acute or late toxicities and both patients obtained complete molecular remission (CMR) after 1 or 6 CTL infusions, respectively. The specific immunological monitoring for quantitation and functional profiling of BCR-ABL-specific T-cell responses was performed on monthly BM and PB samples, by applying IFNg-Elispot and Cytokine Secretion Assays for IFNg, IL-2 and TNFa. In pt.1, BCR-ABL-specific IFNg-producing BM T cells were soon recovered, at low levels (0.20%), after the 1st donor-derived CTL infusion, and then always remained detectable, in association with stable CMR, prevalently showing effector memory (EM) phenotype, both CD4+ and CD8+, and reaching higher frequencies after 6 months (0.86%). Similarly, in pt.2, BCR-ABL-specific IFNg-producing BM T cells, while repeatedly absent during imatinib maintenance therapy, were first detected after the 2nd autologous CTL infusion (0.35%; all EM CD8+) and then raised to higher frequencies (0.90%; prevalently EM, both CD4+ and CD8+), in concomitance with progressive MRD disappearance. In both patients, BCR-ABL-specific BM T cells producing either IL-2 or TNFa were also revealed at several time points. BCR-ABL-specific PB T cells always remained undetectable.

This report shows for the first time that either allogeneic or autologous BCR-ABL-specific CTLs may be safely infused to Ph+ALL patients and may be clinically effective to control MRD, either after disease relapse in post-HSCT setting or during TKI maintenance therapy.

BEST-04

HIGH SENSITIVITY MUTATION MONITORING AND CLONAL ANALYSIS BY ULTRA-DEEP AMPLICON SEQUENCING UNCOVER THE COMPLEXITY OF BCR-ABL MUTATION STATUS IN PHILADELPHIA+ PATIENTS TREATED WITH TYROSINE KINASE INHIBITORS

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Background and Aims. Point mutations in the Bcr-Abl kinase domain (KD) have been implicated in resistance to tyrosine kinase inhibitors (TKI) in chronic myeloid leukemia (CML) and Ph+ acute lymphoblastic leukemia (ALL) patients. Sanger sequencing (SS), the recommended method for mutation screening, cannot identify mutant subpopulations <20%, nor can it discriminate polyclonal from compound mutations, unless it is preceded by cloning. The majority of patients positive by SS have a single mutant clone detectable, more rarely two or more – and multiple mutations are thought to accumulate mainly after multiple lines of therapy. The recent development of massively parallel amplicon sequencing has opened the way to a more accurate qualitative and quantitative characterization of the mutant clones that survive TKI therapy. *Methods.* Longitudinal retrospective analysis of 21 patients with TKI-resistant and 4 patients with TKI-sensitive CML or Ph+ ALL was perfomed with a Roche GS Junior. NGS allowed to screen the Bcr-Abl KD with a lower detection limit of 0.01% and to reconstruct the clonal architecture of the mutated subpopulations, following quantitatively their evolution over time. *Results.* Bcr-Abl mutation status is more complex and dynamic that SS may reveal. In 94% of samples, the higher sensitivity allowed to identify a variety of minor subclones (n=1-8/sample; abundance, 0.35-18.8%) harbouring point mutations, more rarely insertions or deletions. The analysis of patients who later showed evidence of a single TKI-resistant mutation by SS showed that, in all cases, additional low-level mutations were present – either in a small subfraction of the dominant mutated clone, or in an independent one, or both. The analysis of patients who accumulated multiple TKI-resistant mutations as assessed by SS showed that the newly acquired mutations could arise in the pre-existing mutated subclone(s), or in a previously wild-type subclone, but they more often arose in parallel in both wild-type and mutated subclones – generating a complex jigsaw of multiple, competing populations. However, quantitative follow-up showed that only one or a few take over, and some specific compound mutants (T315I+F359V, F317L+M351T) were identified that have greater 'fitness' over single mutants, while others (T315I+E355G; Y253H+E255V) have lower. *Conclusions.* Mutation(s) detectable by SS are often the 'tip of the iceberg'. The Ph+ population is often a mosaic of small mutated subclones. Depending on their absolute and relative 'fitness', some may survive TKI therapy, although only one or a few will be capable to achieve dominance; acquisition of additional mutations dictates further dynamics of shrinkage/expansion. The level of heterogeneity is reduced only transiently when a highly-resistant subclone takes over. Reasoning on the basis of the mutation(s) detectable by SS may not always be sufficient to predict responsiveness to a TKI. Supported by Fondazione Carisbo, PRIN, AIL.

BEST-05

HYPOXIA-INDUCIBLE FACTOR (HIF)-1 IS A THERAPEUTIC TARGET IN MYELOMA-INDUCED ANGIOGENESIS AND BONE DESTRUCTION *IN VITRO* AND *IN VIVO*

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Introduction. The potential role of HIF-1 as a therapeutic target in MM is not known being currently under investigation. We previously reported that bone marrow (BM) microenvironment is hypoxic in multiple myeloma (MM) patients and that HIF-1 is overexpressed by CD138+ MM cells modulating the transcriptional and pro-angiogenic profiles of MM cells. *Methods.* We explored the effect of a stable HIF-1 inhibition in MM cells on proliferation, survival and transcriptional and pro-angiogenic profiles of MM cells either *in vitro* or *in vivo* in NOD/SCID mice. Anti-HIF-1 Lentivirus shRNA pool was used for HIF-1 stable knock-out in human myeloma cell lines (HMCL)s whereas the pKLO.1 lentiviral vector was used as the empty control vector. HMCLs have been infected and selected with puromycin. Selected clones were screened for HIF-1 , HIF-1 , HIF-2 and HIF-3 . The transcriptional profiles were evaluated in the HMCL JJN3 cells transduced with shRNA anti-HIF-1 (JJN3-anti-HIF-1) and on those infected with the control vector pKLO.1 (JJN3-pKLO.1) by U133 Plus2.0 Arrays (Affymetrix®) either in hypoxic or normoxic conditions. Microarray data were further validated by quantitative real time PCR. Finally the effect of HIF-1 inhibition in MM cells was assessed *in vivo* in NOD/SCID mice both in a subcutaneous and intratibial models. Together with tumoral volume and weight, microvascular density was evaluated by CD34 immunostaining. Cortical bone thickness was checked by microQcT in the intratibial mouse model. *Results.* A selective inhibition on HIF-1 expression was observed both in normoxic and hypoxic conditions in HMCls infected with the shRNA anti-HIF-1 as compared to the control vector. Interestingly we found that MM cell proliferation and survival were not significantly suppressed by HIF-1 inhibition neither in hypoxia nor normoxia. Among the genes significantly modulated by HIF-1 inhibition (327 and 361 genes in hypoxic and normoxic condition, respectively) we found that the pro-angiogenic molecules VEGF, IL8, IL10, CCL2, CCL5, MMP9 were down-regulated in JJN3-anti-HIF-1 as compared to JJN3-pKLO.1 Interestingly some pro-osteoclastogenic cytokines were also inhibited such as IL-7 and CCL3/MIP-1 . In the *in vivo* mouse models, we found that the mice, which were injected both subcutaneously and intratibially with JJN3-anti-HIF-1 , showed a dramatic reduction of the weight and volume of the tumoral burden than mice inoculated with the JJN3-pKLO.1. A significant reduction of the number of vessels X field and VEGF immunostaining were observed in both mouse models. Moreover in the intratibial experiments HIF-1 inhibition significantly blocks MM-induced bone destruction. *Conclusions.* Overall our data indicate that HIF-1 suppression in myeloma cells significantly blocks MM-induced angiogenesis and reduces the MM tumoral burden and bone destruction *in vivo* suggesting that HIF-1 is a potential therapeutic target in MM.

ORAL COMMUNICATIONS

Acute Leukemias 1

CO-001

IMMUNOPHENOTYPIC AND MOLECULAR FEATURES OF "CUPLIKE" AMLS

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Nuclear invaginations, also referred to as fishmouth or cuplike nuclei, are often identified in microgranular APL, myelomonocytic and monocytic AMLs. More recently, this typical morphological feature has been associated with NPM1 and Flt3 mutations, as well as with the lack of CD34 or HLA-DR expression, in non APL and non myelomonocytic or monocytic AMLs. In this study we retrospectively analyzed the morphologic, immunophenotypic, cytogenetic and molecular features of 68 AML patients admitted to our Institute since January 2010. A cuplike morphology was defined by the presence of >10% of blasts showing nuclear incision spanning >25% of the nuclear diameter at May Grunwald-Giemsa staining, independently assessed by two hematologists. Four-color or 6-color flow cytometry immunophenotypic analysis was performed on bone marrow aspirate using a FACSCanto II with a large panel of monoclonal antibodies; blasts were gated for CD45 expression and light side scatter characteristics. PCR was used to screen for Flt3-ITDs. NPM mutations were detected by immunohistochemical analysis of abnormal localizations in the NPM protein cytoplasm. Conventional cytogenetics was performed on bone marrow aspirate using standard Giemsa trypsin G-banding procedures. Sixty-eight patients were included in the study, 39 males and 29 females, with a median age of 64 years (26-84). According to FAB criteria, 9 cases were classified as M0, 15 as M1 and 44 as M2. A cuplike nuclear invagination was detected in 15 (22%) cases; the cuplike blasts showed a relatively scanty, pale basophilic cytoplasm with little granularity; there was a 38% median percentage of blast cells with nuclear invagination (range: 10-80). No difference was observed between cuplike and control cases as regards age, sex, FAB group, WBC and platelet counts, haemoglobin level, percentage of blasts and normal karyotype. Loss of CD34 expression was observed in 53% of cuplike cases vs. only 23% in the control group (p: 0.03); the same significant difference was observed for the lack of HLA-DR expression (43% vs. 13%, p: 0.03). In cases with a cuplike morphology, a higher incidence of internal tandem duplication of the Flt3 gene and abnormal cytoplasmic localization of NPM protein were observed (73% and 50% vs. 31% and 24%) but the difference was statistically significant only for Flt3-ITD (p: 0.02). Our data show that non promyelocytic-non monocytic AMLs with cuplike nuclear invagination are associated with Flt3-ITD positivity as well as loss of CD34 and HLA-DR expression. This relationship is not sufficient to suggest that they could represent a distinct AML subtype, but further molecular investigations could yield a better characterization of this AML subgroup.

CO-002

REDUCED FREQUENCY OF THE BCL2-LIKE-10-LEU21ARG VARIANT POLYMORPHISM IN THERAPY-RELATED ACUTE MYELOID LEUKEMIA

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Background. Proteins belonging to the Bcl-2 family are key regulators of apoptosis and have been shown to mediate survival and cell death processes. Bcl2L10 (also named Diva, Boo or Bcl-B) is a member of the Bcl-2 family characterized by an ambiguous function. Specifically, previous independent reports indicated that Bcl2L10 can have both pro- or antiapoptotic functions. The aberrant regulation of apoptosis and cell cycle have been directly linked to many diseases and is one of the hallmarks of cancer. We have previously demonstrated that Bcl2L10 is transcriptionally repressed by promoter hypermethylation and that its overexpression correlates with apoptosis and growth inhibition of the HL60 cell line. Moreover, patients affected by *de novo* and therapy-related acute myeloid leukemia (AML) were more frequently methylated at Bcl2L10 compared to controls. High levels of Bcl2l10 methylation were a negative prognostic factor for 5-azacytidine treatment in patients affected by higher-risk myelodisplastic syndromes (MDS). *Methods.* We performed a case-control study to test the prevalence of the polymorphic variant of the Bcl2L10 gene (Bcl2L10 Leu21Arg; rs2231292) as risk factor for *de novo* and therapy-related AML/MDS (t-AML/MDS). Bone marrow or peripheral blood samples were obtained from 100 *de novo* AML, 94 *de novo* MDS and 85 t-AML/MDS, diagnosed according to the WHO classification. Control peripheral blood samples were obtained from 162 Caucasians with a negative history for previous malignancies. Informed consent was obtained according to institutional guidelines. We established a PCR-RFLP technique to detect the Bcl2L10 Leu21Arg polymorphism (SNP). Hardy-Weinberg equilibrium was calculated for each population and Yates corrected test was used to calculate differences in genotypes population. Odds ratios with 95% confidence intervals were also calculated. *Results.* This is the first report on the frequency of the Bcl2L10 Leu21Arg SNP in myeloid malignancies. Frequencies of the polymorphic variant of Bcl2L10 enzyme were similar in *de novo* AML and controls, whereas there was a trend towards lower frequency of the variant in MDS compared to controls (Table 1). On the other hand, the Bcl2L10 variant was less frequent in t-AML/MDS versus controls (O.R., 0.53; 95% C.I., 0.30-0.94; P=0.027), resulting in a reduced risk for t-AML/MDS. Stratifying the analysis, the frequency of Bcl2L10 variant was lower in t-AML than in *de novo* AML (O.R., 0.42; 95% C.I., 0.18-0.93; P=0.031), whereas no differences were found between *de novo* and t-MDS (data not shown). No significant associations were found between enzymatic polymorphisms and other patients' characteristics, including sex, age, cytogenetics, and therapy of the primary tumor. *Summary and Conclusions.* Bcl2L10 Leu21Arg variant is less frequent in therapy-related AML, compared to *de novo* AML and controls, suggesting a protective role against the development of secondary leukemias.

Table 1.

Genotype	Ref population	Test population	Statistical analysis	
	Controls n (%)	*MDS n (%)*	*OD (95% CI)*	*p-Value*
Bcl2L10-Leu21Arg	162	94		
Leu/Leu	57 (35.2)	44 (46.8)	1.00 (Ref)	
Leu/Arg	85 (52.5)	38 (40.4)	0.58 (0.32-1.04)	0.069
Arg/Arg	20 (12.3)	12 (12.8)	0.78 (0.32-1.89)	0.689
Leu/Arg and Arg/Arg	105 (64.8)	50 (53.2)	0.62 (0.36-1.07)	0.089
	Controls n (%)	*AML n (%)*	*OD (95% CI)*	*p-Value*
Bcl2L10-Leu21Arg	162	100		
Leu/Leu	57 (35.2)	37 (37.0)	1.00 (Ref)	
Leu/Arg	85 (52.5)	50 (50.0)	0.91 (0.51-1.61)	0.827
Arg/Arg	20 (12.3)	13 (13.0)	1 (0.41-2.43)	0.838
Leu/Arg and Arg/Arg	105 (64.8)	63 (63.0)	0.92 (0.53-1.6)	0.869
	Controls n (%)	*t-MDS/AML n (%)*	*OD (95% CI)*	*p-Value*
Bcl2L10-Leu21Arg	162	85		
Leu/Leu	57 (35.2)	43 (50.6)	1.00 (Ref)	
Leu/Arg	85 (52.5)	34 (40.0)	0.53 (0.29-0.97)	0.037
Arg/Arg	20 (12.3)	8 (9.4)	0.53 (0.19-1.43)	0.246
Leu/Arg and Arg/Arg	105 (64.8)	42 (49.4)	0.53 (0.30-0.94)	0.027
	AML n (%)	*t- AML n (%)*	*OD (95% CI)*	*p-Value*
Bcl2L10-Leu21Arg	100	41		
Leu/Leu	37 (37.0)	24 (58.5)	1.00 (Ref)	
Leu/Arg	50 (50.0)	12 (29.3)	0.37 (0.15-0.90)	0.025
Arg/Arg	13 (13.0)	5 (12.2)	0.59 (0.15-2.08)	0.538
Leu/Arg and Arg/Arg	63 (63.0)	17 (41.5)	0.42 (0.18-0.93)	0.031

CO-003

PF-04449913 SPECIFICALLY TARGETS THE HH PATHWAY IN CD34+ CELLS AND REVERTS THE MULTI DRUG RESISTANCE MECHANISM

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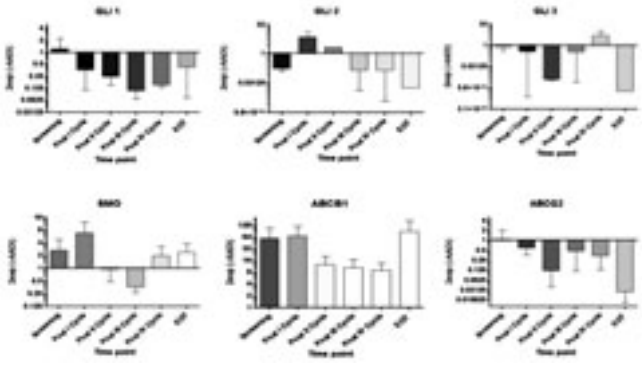
Introduction. Hh pathway activation contributes to leukemia development and growth and its inhibition is likely to offer an efficient therapeutic opportunity.PF-04449913,a Hh pathway inhibitor,is a new selective inhibitor of leukemia self-renewal and is currently being evaluated in phase I clinical trials. *Methods.* In order to identify new potential clinical biomarkers for the PF-04449913,we studied CD34+ LSC collected before and after 28 days treatment in a phase I dose escalation protocol(Clinical Trial Gov. NTC00953758)enrolling selected hematological malignancies (MF,MDS,CML-BP,CMML and AML).We were able to collect and separate highly purified(98%)bone marrow CD34+ cells from 5 AML,1 MF and 2 CML patients (pts) by immunomagnetic separation,and analysed them for GEP (Affimetrix HG-U133 Plus 2.0). *Results.* 1197 genes were differentially expressed between CD34+ cells collected before and after 28 days of PF-04449913 dose finding oral therapy and among these mostly were related to Hh signaling,providing further evidences that PF-04449913 really therapeutically targets the Hh pathway. Regarding genes involved in Hh signaling pathway,Gas1 and Kif27 were strongly upregulated(fold change 1.0947 and 1.12757 respectively;p-value 0.01 and 0.02 respectively)in CD34+ LSC after 28 days exposure to PF-04449913 as compared to baseline,suggesting that they may work as biomarkers of activity. Other genes were differentially expressed after 'ex- vivo' treatment with PF-04449913 as compared to baseline:we observed a down regulation of Bcl2(fold change -1.03004),ABCA2(fold change -1.08966),Gli1(fold change -1.0775), Smo(fold change -1.07702),and an upregulation of Gli2(fold change 1.08191).The GEP results for Gli1,Gli2 and Smo were confirmed by RT-PCR.This analysis by RT-PCR included also Gli3,Abcb1 and Abcg2 genes. To investigate the '*in vitro*'efficacy of Smo Inhibitor we treated MOLM-13,HL-60,KASUMI-1,KG1 ,BV-173,SUPB-15 and K562 cell lines with increasing concentration of PF-04449913(10 nM-100 M)for 24,48 and 72 hours.PF-04449913 had no efficacy on cell viability.Consistent with the WST-1 results, AnnexinV/PI staining analysis did not show efficacy on apoptosis at 24,48 and 72 hours.



EOT: end of treatment.

Figure 1. Gene expression analysis by Real Time PCR.

Conclusions. PF-04449913 specifically targets the Hh Pathway in CD34+ cells, suggesting that Hh inhibition may impair LSC maintenance. New potential biomarkers(Gas1 and Kif27) were identified. *In vitro* treatment with PF-04449913 did not show efficacy neither apoptosis nor in the viability.Taken together, these data may be useful for patients selection strategies and subsequent eradication of the LSC.Therefore, the combination of PF-04449913 with Tyrosine Kinase inhibitors or conventional chemotherapy could represent a valid new therapeutic approach in these haematological malignancies (Figure 1).Work supported by Pfizer, European LeukemiaNet, FIRB 2008, AIRC, AIL, COFIN, University of Bologna and BolognAIL.

CO-004

SEVERAL FUSION TRANSCRIPTS ARE DETECTED BY NEXT GENERATION PAIRED END TRANSCRIPTOMIC RE-SEQUENCING APPROACH IN ADULT BCR-ABL1-POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA (ALL)

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Introduction. Mutations, chromosomal rearrangements and associated gene fusions resulting from inversions, interstitial deletion or translocations represent recurrent findings in leukemia. Nowadays high-throughput "Next Generation Sequencing" technologies and novel developed algorithmic methods allow a unified deep analysis of such alterations with a single base resolution. Taking advantage from these tools, we performed an RNA-Seq (whole transcriptome shotgun sequencing) approach using the Illumina/Solexa platform to define in a single procedure and at high sensitivity the full repertoire of leukemia-related mutations and fusion genes in 3 adult BCR-ABL1+ ALL cases treated with tyrosine kinase inhibitors. *Methods.* Patient median age was 55.3 years (range, 40-70); no additional chromosome abnormalities were detected except for one case with del(9)(p13p22). All the selected cases had previously been profiled by high resolution SNP (Affymetrix SNP6.0) and gene expression (Affymetrix Human Exon 1ST Array) arrays, as well as candidate gene re-sequencing (IKZF1, PAX5, JAK2, CDKN2A, CDKN2B, IDH1, IDH2), lacking missense point mutations. Two cases harbored the deletion of IKZF1, and in one case PAX5 and CDKN2A/B losses were also found. Poly(A) RNA from blast cells was used to prepare Illumina cDNA libraries according to the manufacturer's recommendations. Sequencing by synthesis was performed on an Illumina Genome Analyzer IIx platform, with standard sequencing kits and nucleotide incorporation cycles, generating 75 base pairs (bp) paired end sequence reads. *Results*. A total of 57, 51 and 9 million reads were obtained from the 3 samples and high quality sequence reads were mapped to the reference sequence of the human genome (UCSC hg19) using the Maq software, finding out 58,205, 48,913 and 136,937 putative new single nucleotide variants (SNVs) in the CDS/EXON regions not reported in the dbSNP build 130. Of these, 874 distributed on 290 genes, affected both samples. Thereafter, we analyzed RNA-seq data by deFuse (McPherson A *et al.* PLoS Computational Biology May 2011), a novel computational method for fusion discovery between a gene and an intergenic region. This software uses clusters of discordant paired-end alignments to inform a split read alignment analysis for finding fusion boundaries. First of all, we evaluated the ability of deFuse to rediscovery the BCR-ABL1 gene fusion, then we focused on the most intriguing fusions such as the PBXIP1-PMVK (pre-B-cell leukemia homeobox interacting protein 1- phosphomevalonate kinase) gene fusion. All new gene fusions are under evaluation in a bigger ALL cohort. *Conclusions.* This study provided a comprehensive overview of a BCR-ABL1+ ALL transcriptome, identifying novel mutations and gene fusions involved in Ph+ ALL. Supported by European LeukemiaNet, AIL, AIRC, Fondazione Del Monte di Bologna-Ravenna, FIRB 2006, Ateneo RFO grants, PIO project, Programma di Ricerca Regione–Università 2007–2009.

CO-005

SINGLE-AGENT INHIBITION OF CHECKPOINT KINASE 1 (CHK1) AND 2 (CHK2) BY PF-0477736 (PFIZER) AS A NEW PROMISING THERAPY IN B-ACUTE LYMPHOBLASTIC LEUKEMIA (ALL)

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Introduction. Chk1 and Chk2 are serine/threonine kinases that play a critical role in determining cellular responses to DNA damage both by halting the cell cycle through checkpoint activation and by actively repairing DNA. We explored the cellular effects of single-agent inhibition of Chk1/2 by PF-0477736 and its potential use as a therapeutic strategy for the treatment of B-ALL. *Methods.* Cellular viability was assessed by using a colorimetric assay based on mitochondrial dehydrogenase cleavage of WST-1 reagent (Roche); apoptosis was assessed by use of Annexin V/Propidium Iodide (PI); gene expression profile was performed using Affymetrix GeneChip Human Gene 1.0 ST platform (Affymetrix). *Results.* BCR-ABL1-positive (BV-173, SUPB-15) and negative cell lines (NALM6, NALM19, REH) were incubated with increasing concentrations of PF-0477736 (0.005-2 M) for 24, 48 and 72 hours. Inhibition of Chk1 resulted in dose and time-dependent cytotoxicity with IC50 at 24 hours of 0.1-1.5 μM, with BV-173 being the most sensitive, while NALM6 the most resistant. All cell lines were TP53 wild-type, CDKN2A deleted. Consistent with the viability results, Annexin V/Propidium Iodide staining analysis showed a significant increase of apoptosis at 24 and 48 hours in all cell lines. Functionally, PF-0477736 decreased the inhibitory phosphorylation of Cdc25c Ser216 which is inactivated by Chk1 to prevent mitotic entry and increased the number of H2AX foci, a markers of DNA damage, that culminated in a proportion of cells developing intense staining for H2AX together with nuclear morphological characteristics of apoptosis as demonstrated by immunofluorescence analysis. The efficacy of PF-0477736 was thereafter confirmed in primary blasts from 11 B-ALL patients. Based on the viability results, three groups of patients were identified: very good responders, 46% (IC50: 0.1-0.5 μM at 24 hours); good responders, 36% (IC50: 0.6-1 μM at 24 hours); poor responders, 18% (IC50 > 1 μM at 24 hours). Finally, in order to elucidate the mechanisms of action of PF-0477736 and to determine biomarkers of response, gene expression profiling analysis was performed on treated cell lines and their untreated counterparts (DMSO 0.1%) after 24 hours. Consistent with a specific Chk1-mechanism of action, treatment resulted in differential expression ($p < 0.05$) of genes involved in apoptosis and cell cycle (e.g. CEBPB, CUL1, Histone H1-H2A, 2B family clusters) and DNA damage (DDIT3, GADD34 and GADD45a), suggesting that PF-0477736 contributes to accumulation of DNA damage and subsequent apoptosis in B-ALL cells. *Conclusions.* For the first time we demonstrated the efficacy of PF-0477736 *in vitro* models of B- ALL, suggesting that single-agent Chk1/2 inhibition may be further evaluated in clinical trials. Supported by European LeukemiaNet, AIL, AIRC, Fondazione Del Monte di Bologna-Ravenna, FIRB2006, PRIN2009, PIO program, Programma Ricerca Regione-Università 2007–2009. PF-0477736 provided by Pfizer.

CO-006

IKZF1 DELETIONS ARE MARKERS OF ADVERSE PROGNOSIS IN PEDIATRIC BCP-ALL BCR-ABL NEGATIVE

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Introduction. In the AIEOP-BFM ALL 2000 study, the MRD-based risk groups stratification allowed to achieve more than 80% cure rate. However, relapse is still the most frequent adverse event, occurring mainly in the largest subgroup of non-high risk (non-HR) patients. This emphasizes the need for new prognostic markers for upfront identification of patients with a high risk of relapse. Recently, deletions of Ikaros (IKZF1) gene, key transcription factor in lymphocyte development, have been reported not only in BCR-ABL positive patients, but also in a subset of BCP-ALL patients without known chromosomal aberrations. In this study we analyzed the incidence and prognostic impact of IKZF1 deletions in a representative cohort of 410 non- Down syndrome and BCR-ABL negative BCP-ALL patients enrolled in Italy into AIEOP-BFM ALL2000 study from February 2003 to July 2005. *Methods.* Multiplex Ligation-dependent Probe Amplification (MLPA) (Salsa MLPA P335-A3 ALL-IKZF1 kit; MRC-Holland, Amsterdam, NL) was performed to identify IKZF1 deletions together with deletions in additional B-cell development genes. Patients positive for IKZF1 deletions were further analyzed by Salsa MLPA P202-A1 IKZF1 kit to confirm and better define the extension of the IKZF1 gene deletion. *Results.* IKZF1 deletions were detected in 54/410 cases (13.2%), in keeping with incidence data reported in the literature. In 23 cases (5.6%) the deletion was intra-genic, involving only some exons of the IKZF1 gene, while in 31 cases (7.6%) the deletion was encompassing the whole IKZF1 gene. Patients with deletions of IKZF1 showed a cumulative incidence of relapse (CIR) to 5 years higher than negative patients (24 ± 5.9% vs. 13.1 ± 1.8%, P= 0.049). Moreover, IKZF1 deletions also proved to be a marker of poor outcome when patients with the favorable factor t(12;21) were excluded from the analysis. None of the 8 patients with deletions of IKZF1 belonging to the Standard Risk (SR) group relapsed, vs 10/42 cases (23.8%) stratified in the Intermediate Risk (IR) group and 3/4 cases in the HR group. Five out of 41 patients positive for IKZF1 deletions carried also P2RY8-CRLF2 fusion, another marker of poor diagnosis identified recently in BCP-ALL. Interestingly, 4/5 cases positive for both IKZF1 deletions and P2RY8-CRLF2 fusion relapsed. *Conclusions.* IKZF1 deletions are associated with poor prognosis in BCP-ALL patients (BCR-ABL negative) treated according to the protocol AIEOP-BFM ALL2000 and theirs use as new markers of stratification must be considered.

CO-007

BCR-ABL KINASE DOMAIN MUTATION MONITORING BY ULTRA DEEP AMPLICON SEQUENCING MAY ANTICIPATE EMERGING INHIBITOR-RESISTANT MUTATIONS IN PHILADELPHIA-POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA PATIENTS

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Background. Bcr-Abl kinase domain (KD) mutations and resistance to tyrosine kinase inhibitors (TKI) are a critical problem in Philadelphia-positive (Ph+) acute lymphoblastic leukemia (ALL) patients. Technologies allowing massively parallel, ultra-deep sequencing (UDS) are currently being evaluated in a diagnostic setting since they may conjugate throughputness, sensitivity and accurate quantitation of mutated clones. *Aims*. We tested the ability of an ultra-deep amplicon sequencing strat-

egy for Bcr-Abl KD mutation screening to highlight emerging clones harbouring critical mutations. *Methods*. A total of 61 samples from seventeen Ph+ ALL patients sequentially treated with two or more TKIs (among imatinib, dasatinib, nilotinib, bosutinib, ponatinib) were selected for this retrospective analysis. All the patients had previously been monitored by Sanger sequencing (SS) and were known to have developed one or multiple TKI-resistant Bcr-Abl KD mutations on treatment. In order to reconstruct the timing and dynamics of mutation emergence, longitudinal re-analysis of monthly RNA samples was perfomed with UDS on a Roche GS Junior. UDS achieved a lower detection limit ranging between 0.05% and 0.1% depending on the relative number of sequence reads per patient obtained in each run. Lower detection limit of SS is routinely 25%. *Results.* Amplicons covering the Bcr-Abl KD could be generated and sequenced in all the samples with measurable Bcr-Abl transcripts. In all the samples known to harbor mutations >20% (lower detection limit of SS; n=24), there was 100% concordance between SS and UDS results. UDS could also detect minor (<20%) mutated subpopulations, either alone (6 samples) or in addition to the dominant mutated population detectable by SS (17 samples), as polyclonal or compound mutations. All TKI-resistant minor mutated subpopulations >1% became dominant in 1-2 months since first detection. Presence of these subpopulations could be detected by UDS in patients with molecularly detectable disease at the time of first Bcr-Abl transcript increase, and in some cases even before. TKI-resistant mutations were not necessarily preexisting at diagnosis or at the time of switchover to an alternative TKI, underlining the importance of regular monitoring. UDS also allowed to follow quantitatively the dynamics of emergence and evolution of these mutated populations, to fully characterize their complexity, as well as to reconstruct their clonal relationships. *Conclusions*. In Ph+ ALL, mutation monitoring is a precious tool in the clinical management of patients. UDS proved to be as reliable as SS, to have comparable costs, and to add precious additional quantitative and qualitative information. Although TKI-resistant populations usually take over very rapidly, in approximately half of the patients monthly monitoring with UDS would have allowed to identify them earlier than SS, thus allowing a more timely therapeutic intervention. Supported by PRIN, Fondazione Carisbo, AIL.

CO-008

IDENTIFICATION AND CHARACTERIZATION OF ASPERGILLUS-SPECIFIC IMMUNE RESPONSES IN LEUKEMIC PATIENTS WITH INVASIVE ASPERGILLOSIS

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Introduction. Studies in mice model of IA and in humans healthy subjects have shown that Aspergillus-specific T-cells producing IFNg are protective, while Aspergillus-specific T-cells producing IL10 are non-protective to IA. None of these studies have evaluated the presence of Aspergillus-specific T cells in patients with IA. *Methods.* In 23 leukemic patients with IA, 17 proven and 6 probable cases, during the course of IA, we have evaluated: 1) the occurrence of specific immune response, 2) the Aspergillus antigens most frequently targeted by protective immune responses, 3) whether such protective T cells may be expanded from the peripheral blood of the same patients. Specific immune responses producing IL10, IFNg, IL4 and IL17A were detected by enzyme linked immunospot (ELISpot) assay and characterized by the cytokine secretion assay (CSA). The antigens used were Aspergillus conidia and the recombinant GEL1p, CRF1p, PEP1p, SOD1p, 1–3glucan, 1–3glucan, and galactomannan. Aspergillus-specific T cells were expanded by short term culture with PEP1p, GEL1p, 1-3 glucan and 1-3 glucan. Cytotoxicity was investigated with the XTT assay. The infection course of IA was divided into 4 phases, defined from t1 to t4, each of fifteen days interval, starting from the radiological diagnosis of IA. *Results.* Elispot assay showed that specific T cells producing IL10 were detected to all the antigens, although their frequencies tended to decrease in the fourth phase of the infection (median SFCs/10^6 PBMCs 175 from t1 to t3 versus 95 at t4). On the contrary, specific T cells producing IFNg progressively increased along the course of the infection (median frequencies 35 SFCs/10^6 PBMCs from t1 to t3; 60 SFCs/10^6 PBMCs at t4) and GEL1p, 1–3glucan and 1–3glucan resulted the antigens eliciting the highest number of protective T cells (median SFCs/10^6 PBMCs 65 for the above mentioned antigens vs 32.5 for the remaining four). Of note, patients with a dismal outcome of IA, compared with patients who resolved the infection, demonstrated the complete absence or the late occurrence of only low frequencies of IFNg producing specific T cells, and, virtually the sole presence of specific immune responses producing IL10. The CSA demonstrated that Aspergillus-specific T cells were not only CD4+ T cells but also CD8+ T cells of either central or effector memory phenotype and that Aspergillus-specific T cells producing IL4 and IL17 could be detected to all the antigens. Aspergillus-specific T cells were expanded in a 13-days cultures from five out of five patients and showed a median lytic activity of 9.45% either at 3:1 or at 5:1 effector/target cells ratios. *Conclusions.* This study describes for the first time the Aspergillus-specific immune responses in patients with proven IA, and identify also the antigens predominantly targeted by protective IFNg T cells, with possible consequences in designing therapeutic strategies of either adoptive cell infusion or vaccine therapies.

Chronic Lymphocytic Leukemia 2

CO-009

RELEVANCE OF CHROMOSOME 2P GAIN IN EARLY BINET STAGE A CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction. Chronic lymphocytic leukemia (CLL) is a clinically heterogeneous disease characterized by recurrent chromosomal aberrations of prognostic significance. Recent studies showed gains of the short arm of chromosome 2 (2p) in a recurrent fraction of CLLs. We investigated the prevalence of 2p gain and its relationship with major prognostic biomarkers in a large prospective series of 287 highly purified early-stage CLLs (Binet A) enrolled in a multicentre observational trial. The genomic and gene expression profiles of 2p region were also analyzed. *Methods.* All patients were characterized by FISH for the most recurrent cytogenetic abnormalities and for the major prognostic markers. Based on our previously genome-wide DNA data (Mosca CCR, 2011), specific 2p BAC clones RP11-606L8 and RP11-368O18 were selected for FISH analysis. Furthermore, genomic and gene expression profiles were performed using Affymetrix GeneChip® Human Mapping 250K Nsp and GeneChip® Gene 1.0 ST Array, respectively. *Results.* Gain of 2p was detected by FISH in 17 patients (6%), a finding confirmed by SNP-arrays. Overall, unfavorable cytogenetic deletions, i.e. del(11)(q23) and del(17)(p13) (P=0.002) as well as unmutated (UM) status of IGHV and ZAP-70 and CD38 positive expression were significantly more prevalent in 2p gain cases (P<0.0001). Furthermore, a significantly higher occurrence of sterotyped subset #1 was observed (P=0.0076). Finally, we performed gene expression profiling in 136 CLLs of our series including 12 patients with 2p gain and identified a number of genes significantly upregulated in 2p CLLs, some of which (STRN, NCOA1, and ROCK2) located at 2p. *Conclusions.* This study involving a large prospective series of untreated early-stage CLLs, confirms that 2p gain is a recurrent lesion in CLL and suggest that it may represent an early lesion. The high prevalence of specific stereotyped profile as well as the major biological and cytogenetic risk markers in patients with 2p gains suggest that it may also represent an adverse prognostic lesion, although this finding remains to be clarified. Finally, our data may provide insights for defining novel candidate CLL-associated genes that may play additional pathogenetic roles in the disease.

CO-010

IDENTIFICATION OF NEW POTENTIAL THERAPEUTIC TARGETS IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA BY REVERSE PHASE PROTEIN ARRAY ANALYSIS

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Introduction. In tumor cells, mapping the expression and the activation state of molecules involved in signal transduction could help in identifying new therapeutic targets and provides a mean to patients risk stratification, as well as offers information on protein-protein interactions. Abnormal functions displayed by tumor cells and their microenvironment are the consequence of protein network alterations mediated by internal and external stimuli. These networks, in turn, propagate the signal from the cell surface to the nucleus determining cell fate. Type B Chronic Lymphocytic Leukemia (B-CLL) is a neoplasia characterized by the clonal accumulation of small mature B lymphocytes due to proliferative activity and to apoptosis resistance. Purpose of this study was to gain information about B-CLL proteome by meanings of Reverse Phase Protein Array (RPPA) technology trying to identify new proteins (phosphorylated or not phosphorylated) or pathways which might contribute to the pathogenesis of this disease with the final goal of defining specific targets for new therapeutic strategies. *Methods.* Protein microarrays allow the expression study of several molecules simultaneously. In this study, we performed RPPA analysis with the final goal to identify aberrantly expressed or activated proteins in 57 B-CLL patients and 11 healthy subjects. Signal transduction pathways were assessed for activation/expression status of several key signaling proteins. Data obtained from RPPA were analyzed with appropriate statistic tests and validated by meanings of western blotting and confocal microscopy analysis. *Results.* RPPA analysis, and the subsequent validation of the obtained results, identified two series of proteins: i) molecules whose expression levels reached statistically significant differences in B-CLL vs normal controls (HSP70 and Smac/DIABLO found overexpressed in B-CLL; activated PARP and activated Caspase-6 overexpressed in normal controls); ii) proteins which, although not reaching full statistical significance, are differentially expressed in B-CLL patients with respect to normal controls and whose altered expression is quite consistent with literature data (HS1, gamma-tubulin, PKC alpha/betaII Thr638/641, p38 MAP Kinase Thr180/Tyr182, NF-kB Ser536 and Bcl2 Ser70 found overexpressed in B-CLL; Src Tyr527 overexpressed in normal controls). *Conclusions.* These data highlight the key role of molecules involved in different transduction pathways, particularly in apoptosis control, in B-CLL patients. In particular, this study identify some molecules, differently expressed in B-CLL versus normal B cells, which could be considered for further studies in view of their clinical, prognostic and pathogenic relevance in B-CLL.

CO-011

SEROLOGICAL PROTEOME ANALYSIS (SERPA) AS A TOOL FOR THE IDENTIFICATION OF NOVEL TUMOR ANTIGENS IN CHRONIC LYMPHOCYTIC LEUKEMIA (CLL)

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Introduction. In this study a serological proteome analysis (SERPA) was applied for the first time to identify novel tumor associated antigens (Ag) capable of eliciting humoral immune responses in patients (pts) with chronic lymphocytic leukemia (CLL). The characterization of circulating CLL-specific antibodies (Ab) and related Ag can provide information

about the transformation process and may lead to the identification of predictive biomarkers and suitable targets for immunotherapy. *Methods.* SERPA was performed in 21 untreated pts. Proteins extracted from purified CLL cells were separated by 2-D electrophoresis to obtain proteomic maps which were blotted with corresponding sera by Western Blot to reveal Ab-based reactivity with autologous proteins. To verify the CLL-specificity of Ab recognition, 7 out of 21 pts' maps were also probed with sera collected from 7 healthy donors (HD). For identification, Ag spots were analyzed by Mass Spectrometry (MS). T cells from 6 CLL pts and 3 HD were stimulated with autologous ENOA-pulsed and control dendritic cells (DC) and evaluated by IFN ELISPOT assay. Ag surface expression was analyzed by flow cytometry. *Results.* Sixteen out of 21 CLL sera (76%) were immunoreactive and produced an overall number of 45 Ag spots. By contrast, sera from HD were significantly less reactive and produced only 3 Ag spots. MS analyses led to the identification of 16 different proteins many of which were recurrently recognized by serum Ab. Sera from 48% CLL pts exhibited reactivity with a protein identified as -Enolase (ENOA). By contrast, none of the HD sera was ENOA reactive. The IGHV mutational status was available in 19 CLL pts: 10 were mutated (M), while 9 were unmutated (UM). Interestingly, ENOA was recognized from sera of 7 out of 10 M pts (70%), but only from sera of 3 out of 9 UM pts (33%). Cytofluorimetric analyses showed that ENOA was undetectable on viable CLL cells' surface, whereas it was translocated on the membrane of apoptotic CLL cells. ENOA ability to induce T-cell mediated immune responses was assessed in 6 CLL pts. ENOA-pulsed DC stimulated autologous T cells to secrete IFN in 4 out of 6 pts (66%). This response was ENOA-specific because it was not induced by unpulsed DC or DC pulsed with an irrelevant protein, and also CLL-specific because IFN release was not induced when T cells from 3 HD were stimulated with autologous ENOA-pulsed DC. Interestingly, in 3 out of 4 (75%) pts with ENOA-induced T-cell responses, ENOA specific serum Ab were detectable by SERPA. By contrast, in pts with unresponsive T cells, also ENOA Ab were undetectable. Correlations with the IGHV mutational status showed that all pts with T-cell based ENOA reactivity were M, whereas unresponsive pts were UM. *Conclusions.* These results indicate that ENOA is capable of eliciting CLL-specific humoral and cellular immune responses. Therefore, ENOA can be considered a promising biomarker and a potential target for immunotherapy.

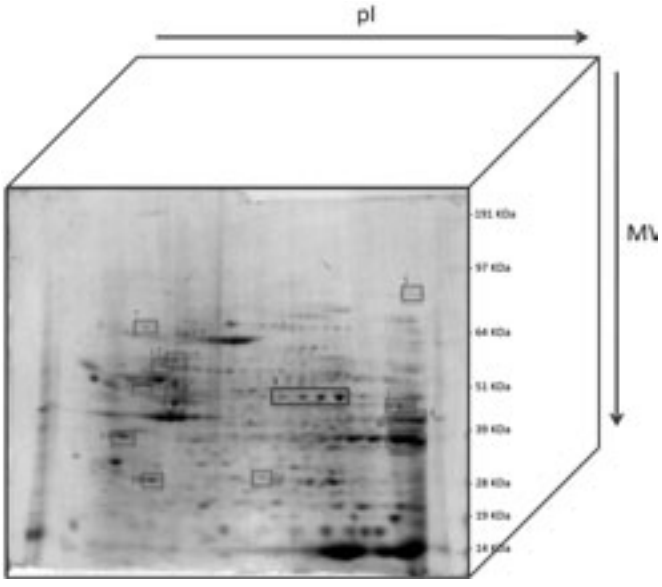

Figure 1.

CO-012

ROLE OF STROMAL CELL-MEDIATED NOTCH SIGNALING IN CHRONIC LYMPHOCYTIC LEUKEMIA SURVIVAL AND RESISTANCE TO CHEMOTHERAPY

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Background. Notch signaling plays an important role in promoting self-renewal of hematopoietic stem cells and in the development of acute lymphoblastic leukemia. Its role in chronic lymphocytic leukemia (CLL) pathogenesis is still unknown. *Aim.* To evaluate the role of the Notch pathway in the survival and chemoresistance of CLL cells in culture with bone marrow mesenchymal stromal cells (BM-MSCs). *Methods.* CLL cells were obtained after informed consent from peripheral blood samples of 12 patients with newly diagnosed CLL, according to the Institutional guidelines. CLL cells and MSCs were studied for the expression of all Notch receptors and ligands. CLL cells were then co-cultured with BM-MSCs at 10/1 ratio for 3 days in absence or presence of Fludarabine, Cyclophosphamide, Bendamustine, Prednisone and Hydrocortisone by adding either anti-Notch-1, -2, -4 or GSI XII for 3 days. Apoptosis of CLL cells was evaluated by Annexin-V/7-AAD staining, while proliferation and cell cycle analysis were assessed by flow cytometry with the CFSE and Propidium methods, respectively. The expression of active Caspase-3, p53, CD23, NF-kB, IL7R, VEGFR2 and Bcl-2 were assessed by flow cytometry. *Results.* Chemotherapeutic agents promoted apoptosis of CLL cells in culture alone, but a consistent rescue from apoptosis of CLL cells was observed when cocultured with MSCs. The blockade of Notch-1, -2 and -4 or all Notch signaling by GSI XII in presence of chemotherapeutic agents dramatically lowered the number of overall live CLL cells even in co-culture with MSCs. Accordingly, active Caspase-3 was overexpressed, whereas Bcl-2, NF-kB and CD23 was weakly expressed in CLL cells in culture alone in presence of chemotherapeutic agents. Active Caspase-3 down-regulation, and Bcl-2, NF-kB, CD23 over-expression in CLL cells were observed following co-culture of CLL cells with BM-MSCs in presence of hydrocortisone. These features were reverted by adding either anti-Notch-1 + anti-Notch-2 + anti-Notch-4 antibodies or GSI XII in presence of chemotherapeutic agents. *Conclusions.* Notch-1, -2 and -4 signaling mediated by BM-MSCs confers resistance to Fludarabine, Cyclophosphamide, Bendamustine, Prednisone and Hydrocortisone by upregulating Bcl-2, NF-kB and CD23 in CLL cells.

CO-013

AUTOCRINE ROLE OF ENDOTHELIN-1 /ETA RECEPTOR SIGNALING PATHWAY IN CHRONIC LYMPHOCYTIC LEUKEMIA: INVOLVEMENT IN SURVIVAL AND INTERACTION WITH ENDOTHELIAL CELLS

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Introduction. The endothelin axis, comprising endothelins (ET-1, ET-2 and ET-3) and their receptors (ETAR and ETBR), has recently emerged as relevant player in tumor growth and metastasis by regulating cell survival, angiogenesis, tumor-infiltrating immune cells, epithelial-to-mesenchymal transition, invasion and metastatic dissemination. Several small molecule antagonists of ET-1 receptors are currently undergoing clinical trial as novel agents in cancer therapy. We investigated whether ET-1/ETAR signaling pathway may represent a novel autocrine loop in chronic lymphocytic leukemia (CLL) B cells. *Methods.* Leukemic cells were purified from peripheral blood of untreated CLL patients and cultured in RPMI/10%FBS in time-course experiments. In co-culture experiments, CLL cells were cultured in complete medium on endothelial cell layers (HC) formed by Human Umbilical Vein Endothelial cells (HUVEC) or Human Microvascular Endothelial cells (HMEC). Apoptosis was evaluated by flow cytometry using Annexin V/PI staining. ET-1 and ETAR expression on CLL cells was evaluated by high density microarray, RT-PCR, ELISA, flow cytometry and immunohistochemical staining of CLL-infiltrated bone marrow and lymph nodes. *Results.* CLL cells circulating in peripheral blood and infiltrating tissues secreted ET-1 and expressed

ETAR on the cellular surface. When CLL cells were cultured alone in complete medium, the addition of recombinant ET-1 peptides at 1nM, 10nM and 100nM determined a 4.1, 5.9 and 6.7-fold increase in CLL survival relative to control (CLL without treatment) (P<0.05, Wilcoxon test). The blockage of ETAR throughout BQ 123 selective antagonist abrogated the ET-1 mediated apoptosis resistance. Moreover, after co-culture on endothelial cell layers, CLL cells expressed higher levels of ET-1 as discovered by high density microarray analysis (n=9, FC=9.2, P=0.006) and validated by RT-PCR (n=30, mRNA Relative Quantity, 2.6±0.8 at baseline, 5.1±1.2 in culture alone for 72h, 189.1±43.2 in HC for 72h, P<0.05). In agreement, we also found a huge increase in ET-1 secretion from 0.6±0.1 pg/mL in CLL alone to 51.6±0.5 pg/mL in HC system (n=26, P<0.05, Mann-Whitney test). As consequence, we argued whether ET-1/ETAR axis could be involved in survival advantage mediated by endothelial cells. We found a 2.5-fold increase in CLL mean relative viability (MRV) in HC compared to cells cultured alone (Figure 1). Pretreatment of the cells with 0.1 and 1 μM BQ 123 significantly reduced MRV in HC condition to 1.7 and 1.4-fold change compared to cells cultured alone (n=11, 72h,*P<0.05) (Figure 1). *Conclusions.* Our findings suggest a role of ET-1 signaling via ETAR in prolonged survival of CLL cells. CLL survival advantage obtained by interaction with endothelial cells is partially mediated by activation of ET-1/ETAR axis.

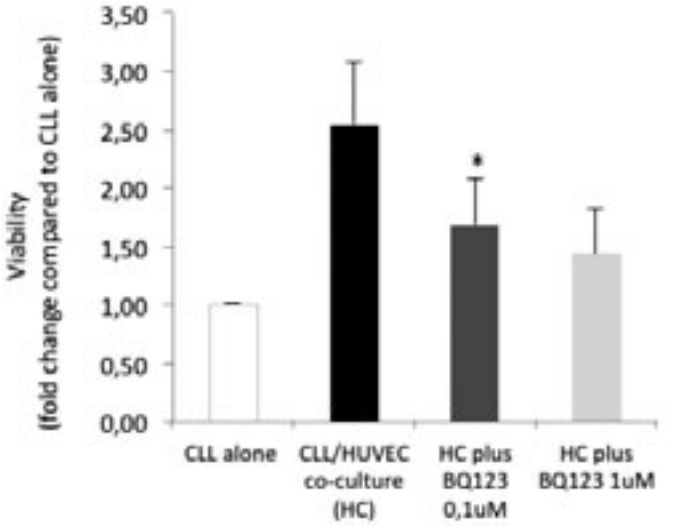

Figure 1.

CO-014

MICRO-RNA AND GENE EXPRESSION PROFILING IN CHRONIC LYMPHOCYTIC LEUKEMIA-LIKE (CLL-LIKE) CLINICAL MONOCLONAL B LYMPHOCYTOSIS AND RAI-0 CLL: A COMPARATIVE ANALYSIS OF A PROSPECTIVE MULTICENTER OBSERVATIONAL STUDY

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Introduction. It is still unclear whether cMBL, defined by the B-lymphocyte count, represents a biologically different category from Rai0-CLLs. *Methods*. 463 Binet stage A patients entered the prospective O-CLL1 protocol (clinicaltrial.gov identifier NCT00917540); 352 were staged as Rai0 and were included in this study; of these, 136 cases were cMBL. GeneChip® Gene 1.0 ST Array and G4470A Agilent Human miRNA microarray were used. *Results*. cMBL showed only a significantly low number of IGHV-UM cases, while there was no difference in CD38 or ZAP-70 expression, or in type and incidence of genetic aberrations by FISH. Noteworthy, Rai0-CLL and cMBL cases with CD38 and ZAP-70neg or M IGHV had a similar risk of PFS suggesting that other biomolecular features could have a role. Thus, GEP and miRNA analyses were performed on purified B-cells from 160 CLL cases. Unsupervised evaluation of GEP data indicated that cMBL cases were intermingled among Rai0-CLL cases, thus suggesting similar transcriptional patterns. In addition, miRNA global profiling showed no specific grouping of cMBL cases by unsupervised analysis. Given this evidence, we next applied a supervised approach in the attempt to identify genes/miRNAs distinguishing the two groups. However, no specific signatures were identified in both cases even at a relatively high stringency level; only a single transcript (Loc400986, down-regulated) and miRNA (miR130a, up-regulated) were found to be differentially expressed between cMBL and CLL-Rai0 patients, respectively. Notably, miR-130a has been recently reported to be involved in CLL cell survival by regulating the activity of other miRNAs. A multiclass supervised analyses of GEP and miRNA data comparing 4 patient groups (UM/M cMBL and UM/M Rai0-CLLs) identified a large array of genes/miRNAs, the majority of which showed a similar modulation pattern in UM vs M cases in both cMBL and CLL-Rai0. However, when a two-class analysis was performed (i.e., comparing M vs UM cMBLs and M vs UM Rai0-CLL), a number of genes/miRNAs were found to be modulated at significant levels either in cMBL or CLL-Rai0 patients, respectively. As an example, miR-29c remained significantly down-regulated in UM vs M CLL-Rai0, but not in UM vs M cMBL cases. Notably, miRNA-146b-5p appeared to be the only miRNA to share a significant down-regulation in UM cases independently of the cMBL status. PFS probability of patients with high level of miRNA-146b-5p was 90% as compared to 83.6% of those with low miRNA (P<.0001). The miRNA-146b-5p prognostic impact was maintained, although with a different statistical power, either in among cMBL (P=.079) or Rai0-CLL (P<.0001) patients. *Conclusions*. Our study indicates that cMBL and Rai0-CLL patients share similar gene and miRNA transcriptional patterns and that differences found were mainly related to the effect of different proportion of UM IGHV cases associated with the two groups. Yet, miRNA analysis could further fine-tune prognosis in early CLL.

CO-015

THE MEVALONATE PATHWAY AS A METABOLIC TARGET TO CIRCUMVENT MULTIDRUG-RESISTANCE IN CHRONIC LYMPHOCYTIC LEUKEMIA CELLS (CLL)

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Introduction. The immunoglobulin heavy-chain variable region (IGHV) mutational status defines two subtypes of chronic lymphocytic leukemia (CLL). Patients carrying unmutated (UM) IGVH suffer from a more aggressive disease than those with mutated (M) IGVH. The tumor microenvironment plays a pivotal role in supporting survival and in vivo accumulation of CLL cells and confer a multidrug resistance (MDR) phenotype to CLL B cells. MDR mainly relies on the over-expression of membrane transporters, like P-glycoprotein (Pgp), which extrudes several anticancer drugs. The Pgp protein is the product of the mdr1 gene whose regulation is under the positive control of the mevalonate (Mev) pathway, the Ras/Rho dependent signaling and the transcription factor HIF-1 . The aim of this study was to investigate the MDR phenotype and signaling cascade in M and UM CLL cells under basal conditions and after exposure to prosurvival microenvironment-mediated signals. *Methods.* M and UM CLL cells were cultured in the presence and in the absence of murine stromal cells (M210B4) and exposed to Zoledronic acid (ZA) (1 mol/L), Simvastatine (1 mol/L), ERK1/2 kinase inhibitor PD98059 (10 mol/L), HIF-1 inhibitor YC-1 (10 mol/L) and Doxorubicine (Doxo) (1 mol/L). The Mev pathway activity was measured by cells radiolabelling with [14C]-mevalonic acid and thin layer chromatography. Ras, ERK1/2 and Akt activity were detected by Western blot. Rho, Rho Kinase and HIF-1 activity were assessed by ELISA. Mdr1 expression was

measured by Real Time-PCR and PgP activity was evaluated by measuring Doxo intracellular accumulation. Doxo cytotoxicity was assessed by annexin V and propidium iodide staining. *Results.* The Mev pathway is significantly more active in UM than in M CLL cells. This hypermetabolic activity translates into a higher activation of Ras/Akt and Rho/Rho kinase signaling pathways and a higher expression of HIF-1 . HIF-1 activation positively regulates mdr1 gene expression in UM cells leading to a more effective Doxo extrusion and therefore to a protection of cell viability upon Doxo exposure. M210B4 further protect UM cells from Doxo induced cell death, by strongly upregulating the activity of the Mev pathway and the activation of HIF-1 /mdr1/PgP axis, and significantly increasing Doxo extrusion. Targeting the Mev pathway of UM cells with ZA or statins or using PD85 and YC1-10, specific inhibitors of ERK-1/2 and HIF-1 , reduces the basal activity of HIF-1 /mdr1/PgP axis and significantly increases Doxo retention and cytotoxicity. These agents are capable to abrogate the protective effect exerted by M210B4, by significantly increasing PgP activity and Doxo-induced cell death. *Conclusions.* These data indicate that the pharmacological inhibition of the Mev pathway and of the downstream HIF-1 /mdr1/PgP axis can be regarded as a novel potential strategy to circumvent basal and environment-mediated chemoresistance of CLL cells.

CO-016

PATTERNS OF GLOBAL DNA METHYLATION IDENTIFY EARLY STAGE (BINET A) CLL PATIENTS WITH HIGHER RISK OF DISEASE PROGRESSION

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Introduction. Studies on global DNA methylation in B-cell chronic lymphocytic leukemia (CLL) are still limited. In the present study, we integrated the investigation of methylation status of the genome with the global transcriptional data. *Methods.* The methylation profiles of 37 Binet-A CLL patients from an Italian multicenter study were generated on Illumina Human Methylation27K arrays. The signals of the examined 27578 CpG sites were summarized by the M-value, defined as the log2 ratio of the intensities of methylated versus unmethylated probes. The differential methylation levels of CpG sites were evaluated by Significance Analysis of Microarrays algorithm. A publicly available dataset (Kanduri *et al*., 2010) was used for meta-validation analysis. To assess inverse correlation between methylation status and mRNA expression, we applied Kendall's tau statistic to M-values and the corresponding transcriptional data generated on GeneChip® HuGene 1.0ST arrays. For each CpG site, globaltest procedure in R software was used to test the association of M-value and disease progression assesses as time to the first treatment (TFT). *Results*. Global methylation analysis indicated that the CLL samples were mainly clustered based on IGHV mutational status. Supervised analysis identified a methylation pattern of 46 up- and 178 down-methylated probes that characterize the 18 IGHV unmutated (UM) and the 19 mutated (M) patients. Among them, the RIPK3, ISG20L2, ABI3, IMP4, MCM5, GPR55, RAB24, AYTL2 genes showed the largest modulations from a strong hypomethylated status in UM samples to hypermethylated status in M samples, whereas CST7, CLDN15, LOC340061, PECAM1 and WISP3 had the opposite behavior. Most of the best-ranked genes showed consistent profile with previously published study (Kanduri et al, 2010). To identify whether the methylation status of a group of genes might describe the clinical outcome, we performed a selection of the CpG islands whose M-values were associated with TFT at higher extent ($P<0.01$ using globaltest). In this way, we derived a 53-gene signature that clustered the CLL patients into 3 groups with different prognosis ($P=1.13e-06$). In line with previous reports, the group with the poorest outcome carried UM IGHV, ZAP70high and CD38high profiles. Finally, the integrated analysis of methylation and transcriptional data revealed 36 probes (corresponding to 30 genes) whose methylation status was inversely correlated (p-adjusted <0.05) with the corresponding expression levels. Interestingly, nine of those probes (specific for PHYHD1, ZNF471, ICOS, SLC16A4, PRICKLE2, ZNF135, SSH3, and two for C8orf31 genes) were also included in the 53-gene signature associated with outcome. *Conclusions.* Our global methylation analysis revealed genes that may have implications in the biology and prognosis of CLL, and strongly suggested that the methylation pattern might be used as surrogate to identify CLL cases with higher risk of progression.

Stem Cell Transplantation

CO-017

MICROPOROUS BIPHASIC CALCIUM PHOSPHATE GRANULES (MBCP+®) RETAIN IMMUNOLOGICAL PROPERTIES OF BONE MARROW-DERIVED MESENCHYMAL STROMAL CELLS AND PROMOTE OSTEOBLASTIC DIFFERENTIATION

Bassi G,[1] Guilloton F,[3] Ménard C,[2] Pacelli L,[1] Di Trapani M,[1] Carusone R,[1] Bifari F,[1] Sensebé L,[3] Deschaseaux F,[3] Baroth S,[4] Schrezenmeier H,[5] Layrolle P,[6] Tarte K,[2] Krampera M,[1] on behalf of reborne consortium (7th framework programme of european commission: ga n° 241879)

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Introduction. Bone is among the most frequently transplanted tissue with about 1 million procedures annually in Europe. Despite their considerable disadvantages, allografts and autografts account for more than 80% of total graft volume. Significant growth opportunities exist for synthetic bone grafts in association with mesenchymal stromal cells (MSC) from autologous or allogeneic sources as alternatives to biological bone grafts in orthopaedic and maxillofacial surgery. The objective of REBORNE is to perform clinical trials using advanced biomaterials and cells triggering bone healing in patients. Aim of the Immunological Unit of Reborne is to assess the MSC immunomodulatory properties in the presence of the biomaterial (MBCP+®, Biomatlante) used as scaffold for MSC delivery. *Methods*. Bone marrow MSC were provided from REBORNE Consortium Centres. To perform proliferation assays, different immune effector cells (T, B and NK cells) were stained with CFSE according to manufacturer's protocol. For quantification of survival of immune effector cells after co-culture experiments we set up active caspase-3 cell staining. Differentiation potential was evaluated by culturing MSC with two different media containing either bone morphogenetic protein 4 (BMP4) or dexamethasone. After three weeks, osteogenic differentiation was quantified by qRT-PCR, alkaline phosphatase activity and alizarin red staining. *Results.* We found that primed MSC, pre-treated with the inflammatory cytokines IFN and TNF, displayed upregulation of HLA-ABC, CD54, CD106 and *de novo* expression of HLA-DR, both in standard culture conditions and in association with MBCP+®. No significant differences were found between standard coculture conditions and 3D-coculture conditions, in terms of proliferation of immune effector cells. We demonstrated that, in both experimental conditions, resting MSC suppressed T and NK cells proliferation and this effect was stronger after priming with inflammatory cytokines. In contrast, B cell proliferation was inhibited only in co-culture with primed MSCs, with slight differences related to the culture system. We also showed that immune effector cells viability was not affected by the biomaterial and that MSC coculture increased their survival even in presence of MBCP+®. Dexamethasone and BMP4 were capable of inducing MSC differentiation into osteoblast-like cells, as confirmed by qRT-PCR analysis. We demonstrated that BMP4-based medium led to fully differentiated osteoblast (Osterix+, RUNX2+, DLX5+ and alkaline phosphatase+). Moreover, MBCP+® was more efficient in increasing osteoblastic differention as compared to standard culture conditions, as shown by the higher expression of Osteocalcin and Osterix. *Conclusions*. These data show that the association of MBCP+® and MSC does not affect MSC properties and suggest that it could be a treatment of choice of bone defects instead of allograft and autograft transplantation.

CO-018

NONINVASIVE PREDICTION OF ACUTE GRAFT-VERUS-HOST DISEASE (AGVHD) BY CIRCULATING MIRNA PROFILING

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Introduction. Despite substantial progress in the understanding of the complex immunobiological processes involved in HSCT and the recent advances in post-transplant immunosuppressive therapy, aGVHD still represents the major life threatening complication resulting in poor outcome. There is no validated diagnostic test for aGVHD, thus the identification of biomarkers with diagnostic and possibly prognostic significance might eventually favourably impact the transplantation outcome. We hypothesized that the prospective analysis of miRNA expression profile in the plasma of allografted patients could allow for the detection of specific miRNAs with predictive role for aGVHD. *Methods.* After informed consent, we collected plasma samples from 10 healthy donors and 24 patients (median age: 59 and 41 years) who received unmanipulated HSCT (19 from Matched Unrelated Donors and 5 from HLA-matched siblings). Blood samples were collected weekly after HSCT and patients were monitored to assess aGVHD onset. Four of 24 patients developed intestinal GVHD (grade 2) while 10 of 24 patients developed cutaneous GVHD (grade 2-3). MicroRNAs were isolated from plasma and miRNA expression profile examined using a quantitative PCR-method (TaqMan® Human microRNA Cards, Applied Biosystems). Cts above 35 in at least 75% of the patients were excluded from subsequent analysis. Data were normalized by performing reciprocal ratios of miRNA expression values. The obtained ratios were used to develop a classification algorithm using Support Vector Machines (SVM), combined withMann-Whitney test for the identification of relevant miRNAs. The performance of the algorithm was evaluated using leave one out cross-validation. *Results.* The SVM algorithm was tested using up to 50 miRNA ratios (sorted by p-values) in order to select the best model for GVHD prediction. Results showed that a panel of 11 miRNAs (8 ratios, 11 unique miRNAs) was able to discriminate between patients that would develop aGVHD from those without aGVHD with an accuracy of 0.87, sensitivity of 0.92 and specificity of 0.80. Pathway enrichment analysis performed using DIANA-mirPath software on the target genes, predicted by microT-4.0 indicate that the miRNAs identified with the SVM approach regulate critical pathways of GVHD pathogenesis (TGF-beta and Wnt signaling, T cell receptor signaling, Jak-STAT pathway). *Conclusions.* Considering the nonivasive characteristics of plasma sampling and the reproducible and easy detection of circulating miRNAs, our results show that it is possible to identify a panel of miRNAs with prognostic significance. The new approach of normalization seems to be very robust and overcomes the quantification issues of circulating miRNAs. Despite the small number of patients the development of an SVM-based algorithm allows the prediction of aGVHD onset with very good performances. Nevertheless a validation of the present predictor will be carried out on an independent set of patients.

CO-019

IN VIVO AND *IN VITRO* CHARACTERIZATION OF BONE MARROW-DERIVED CD133+ PLURIPOTENT STEM CELLS AS CELLULAR THERAPY IN CHRONIC LIVER FAILURE

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A phase I safety trial of cellular therapy in patients with end-stage liver disease (ESLD) is currently ongoing in our Institution. Highly purified, autologous G-CSF-mobilized CD133+ cells are reinfused through the

hepatic artery by transfemoral or transbranchial arteriography. CD133+ cells are administered to patients starting from 5x104/Kg patient's body weight and increased every 3 patients. The maximum infused cell dose is $1x10^6$/Kg. G-CSF at 5 µg/Kg/day is administered sc for 3 days after the reinfusion of stem cells (SCs). Up to date, 13 patients with ESLD have been mobilized with G-CSF and highly purified autologous CD133+ SCs have been reinfused in 10 patients. No adverse events have been recorded during mobilization or intrahepatic SCs reinfusion. Biological studies on mobilized SCs show that in patients with ESLD: 1) highly purified CD133+ SCs have hemopoietic and endothelial potential; 2) at baseline, the number and the clonogenic capacity of circulating hematopoietic and endothelial progenitors are significantly reduced as compared with the normal counterparts. However, they are markedly increased after G-CSF treatment together with the serum concentration of HGF, VEGF and MMP9. To further investigate the potential role and the mechanisms of action of BM-derived SCs therapy in cirrhosis we established a murine model of chronic liver injury and fibrosis. Specifically, C57BL/6N mice received CCl4 by inhalation for thirteen weeks and were treated with Cyclosporin-A. Transplantation was performed by tail vein injection of 106 CD133+ SCs from three cirrhotic patients. After four weeks from transplantation all mice were sacrificed. We demonstrated that mice transplanted with CD133+ human SCs appear to have less fibrotic septa than mice without SC transplantation, suggesting the potential therapeutic role of human SCs on the recovery of liver fibrosis. Moreover, we characterized the bi-directional effects of the co-cultures of human G-CSF-mobilized CD133+ SCs with the LX-2 human hepatic stromal (stellate) cells. We show that, in cell-cell contact cultures, LX-2 cell line significantly increases the survival of CD133+ SCs with the concomitant reduction of apoptosis. In addition, the hemopoietic clonogenic capacity of CD133+ SC is significantly increased. Therefore, the liver microenvironment contributes to the survival and differentiation of CD133+ SCs. Taken together, these results suggest that CD133+ SCs have the potential to improve liver function.

CO-020

HIGH-DOSE RITUXIMAB IN THE CONDITIONING REGIMEN BEFORE ALLOGENEIC STEM CELL TRANSPLANTATION FOR RELAPSED LYMPHOMAS: PROTECTIVE ROLE OF THE ANTIBODY ON ACUTE GVHD INCIDENCE

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Introduction. Allogeneic stem cell transplantation (alloSCT) with a reduced-intensity conditioning (RIC) is an effective salvage therapy for relapsed lymphomas. The addition of Rituximab (R) in the conditioning could enhance the anti-lymphoma effect. The high-dose R before alloSCT was studied by MD Anderson group in indolent (LG), but not in aggressive (HG) lymphomas. We designed a prospective, multicenter, phase II study. *Objective*. Primary end-point was 1-year progression-free survival; secondary endpoints were non-relapse mortality, incidence of acute and chronic graft-versus host disease (GVHD). *Methods*. Treatment plan consisted of high-dose R (500 mg/ms), thiotepa (12 mg/kg), fludarabine (60 mg/kg) and cyclophosphamide (60 mg/kg). GVHD prophylaxis included cyclosporine and short course methotrexate; ATG (7 mg/kg) was added for pts allografted from one antigen mismatched sibling or unrelated donors. Fifty-four pts are now evaluable. Histopathological subtypes included 25 HG (n= 18 diffuse large B-cell lymphomas, n= 7 mantle cell lymphomas) and 29 LG (n=15 follicular lymphomas,n=14 chronic lymphocytic leukemia). Pts were allografted from related siblings (SIB) (n= 34 matched, n=1 one mismatched) or unrelated donors (UD) (n=15 matched, n=4 mismatched). Ten of 25 pts (40%) with HG and 11 of 29 pts (38%) with LG were in complete remission at time of allo-SCT. *Results*. At median follow-up of 21 months (range, 5-52 months), 40 pts are alive and 14 died: 6 for non-relapse mortality (NRM), and 8 for disease. The cumulative incidence (CI) of NRM was 12% at 1 year [9% versus 16% for alloSCT from SIB and MUD (P=0.3)]. In total only 13 of 54 patients had acute GVHD (n=10 grade II, n=3 grade III) with an estimated CI of 23% at 100 days. Interestingly, acute GVHD was inferior in pts allografted from MUD donor where the ATG and R combination was used (11% versus 29% from MUD and SIB donors, respectively). The main infectious complications were: n=4 (8%) sepsis, n=10 (19%) pneumonia, n=3 (6%) viral (no CMV), n=2 (4%) fungal. The median value of CD19+ at 1 year was 148 cells/ucl (93% IgD+CD27-, 5% IgD+CD27+, 2% IgD-CD27+).The CI of relapse at 3 years was 38% and 51% in LG and HG lymphomas, respectively. The 2-years OS and PFS were 83% and 61% for LG and 63% and 34% for HG lymphomas, respectively. *Conclusions*. We observed: 1) reduced incidence of acute GVHD especially in pts receiving a combination of ATG and R; 2) the outcome of pts affected by HG was not improved by the administration of R.

CO-021

PENTRAXIN-3 AS GRAFT-VERSUS-HOST DISEASE (GVHD) BIOMARKER IN A PEDIATRIC COHORT OF HEMATOPOIETIC STEM CELL TRANSPLANTED PATIENTS

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Background. GvHD is a major obstacle to safe allogeneic haematopoietic stem cell transplantation (HSCT). Reliable biomarkers facilitating the early and accurate recognition of this invalidating disease are highly warranted to improve its management. The long pentraxin (PTX)-3 is locally produced at sites of inflammation. Its rapid increase in different human inflammatory diseases and the correlation between PTX-3 levels and disease severity suggest the potential usage of this molecule as GvHD diagnostic marker. *Methods*. We collected plasma samples from 71 pediatric patients, who received HSCT at S. Gerardo Hospital, Monza, before the beginning of the conditioning regimen and weekly from the transplant day (day 0), until day 100. After HSCT, 33 patients developed acute GvHD very early within day 28, 21 developed GvHD between day 28 and 100, while 17 never developed it in the monitored time-frame. Concerning GvHD patients, blood samples were further collected at the day of GvHD onset, before the beginning of GvHD-specific drug therapy. PTX-3 plasma levels were monitored by ELISA. *Results*. Firstly, to investigate the possible usage of PTX-3 as GvHD marker, we evaluated its plasma levels at disease onset. Since PTX-3 values could be influenced by the occurrence of infections, we compared PTX-3 levels measured at the onset of GvHD before day 28 after HSCT, time-frame always resulting free from infectious events in our cohort of patients, with time-matched PTX-3 levels measured in patients who did not develop GvHD within day 28 (no GvHD group). The median PTX-3 level at the onset of GvHD was 33.4 ng/ml (range=11.2-847.4), significantly higher than the no GvHD group (median PTX-3 level=14.4, range=4.2-58.5 ng/ml). The difference between the two groups was statistically significant, Wilcoxon p-value<0.0001. Secondly, to investigate its predictive potential, we further compared PTX-3 levels at day 0, 7 and 14, normalized for each patient by his baseline (PTX-3 value before conditioning regimen), between patients with early GVHD occurrence (within day 28) and patients of the no GVHD group. We observed that the conditioning regimen induced an increase of PTX-3 levels compared to the baseline in both groups, without significant differences. On the contrary, very interestingly, we noted that at day 7 and 14 the median PTX-3 level resulted significantly higher in patients experiencing GVHD within day 28 (PTX-3 normalized change= 3.03) compared to the no GVHD group (PTX-3 normalized change= 1.1; Wilcoxon p-value= 0.05). *Conclusions*. These preliminary results show that PTX-3 plasma levels increase very rapidly in patients experiencing acute GvHD, candidating this molecule as an easily measurable soluble factor useful to corroborate the clinical monitoring of the pathology. Furthermore, if confirmed in a larger cohort of patients, PTX-3 could represent a sensitive biomarker able to predict patients at high risk for developing GvHD early after HSCT.

CO-022

COMBINED THERAPY WITH SONIC HEDGEHOG GENE TRANSFER AND BONE MARROW-DERIVED ENDOTHELIAL PROGENITOR CELLS FOR PERIPHERAL LIMB ISCHEMIA

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Introduction. We have previously demonstrated that Sonic hedgehog (Shh) gene transfer improves angiogenesis, arteriogenesis, and vasculogenesis in the setting of ischemia, by up-regulating the expression of multiple growth factors and enhancing the mobilization and homing of endogenous bone marrow (BM)-derived endothelial progenitor cells (EPCs). Moreover, Shh gene therapy enhances the incorporation of circulating BM-derived EPCs into the growing neovasculature. *Methods*. Here, we investigated whether the effectiveness of Shh gene therapy could be improved by the combined administration of exogenous EPCs. We induced unilateral hindlimb ischemia in old mice, which have reduced angiogenic properties, and treated them with either a plasmid containing the coding sequence of the human Shh gene (phShh), BM-EPCs, or both. Response to treatment was evaluated in terms of capillary density, incorporation of administered EPCs into the site of ischemia, and number of regenerating myofibers. *Results*. We found that capillary density and number of regenerating myofibers were significantly higher in the in phShh + EPC-treated muscles compared to the other experimental groups. We also found that phShh gene transfer increases the incorporation of transplanted EPCs at the level of the ischemic site. *Conclusions*. In summary, combined treatment with Shh gene transfer and BM-derived EPCs more effectively promotes angiogenesis and muscle regeneration than either individual treatment and merits further investigation for its potential beneficial effects in ischemic diseases.

CO-023

NATURAL KILLER CELL EXPANSION UNDER GOOD MANUFACTURING PRACTICE (GMP) CONDITIONS FOR CLINICAL USE IN ACUTE LEUKEMIA PATIENTS

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Introduction. The clinical management of acute leukemia patients has gone through radical changes during the last decade. Different therapeutic protocols (including targeted and biological therapies) have been applied to subgroups of patients characterized by a different prognostic likelihood. The anti-leukemic potential of natural killer (NK) cells has over the years raised considerable interest. We have previously demonstrated the possibility of expanding cytotoxic NK cells with killing activity against autologous blasts from adult and pediatric acute lymphoid leukemia (ALL) and acute myeloid leukemia (AML) patients in complete remission. We now aim at the design of a method of NK cell expansion under good manufacturing practice (GMP) to be used in clinical protocols based on the in vivo infusion of ex vivo expanded autologous or allogeneic NK cells for patients affected by acute leukemia. *Methods.* Peripheral blood mononuclear cells (PBMC) obtained from eight healthy donors were isolated by gradient centrifugation. For NK cell enrichment, a two-step immunomagnetic procedure was used, consisting of an initial CD3+ T-cell depletion followed by a CD56+ positive selection. Isolated NK cells [1 10(5)/ml] were suspended in serum-free medium supplemented with 5% autologous plasma in the presence of autologous irradiated feeder cells [2.5 10(5)/ml], 500 U/ml IL-2 and 50 ng/ml IL-15, and cultured at 37°C for 14 days. Only GMP and clinical grade materials were used. The phenotype of freshly-isolated or expanded NK cells was assessed by flow cytometry on a FACSCanto with anti-human monoclonal antibodies directed against the CD56, CD16, CD3, DNAM-1 and NKG2D receptors, being the last two proteins involved in NK cell recognition and killing of primary blasts. The cytolytic properties of expanded NK cells were tested in a (51)Cr release assay against the K562 and HL-60 cell lines, and against allogeneic AML and ALL primary blast cells. *Results.* NK cells presented a 34±16 fold increases after a 14 day culture period. Flow cytometric analyses revealed that the ex vivo-generated NK cells contained a homogenous cell population displaying a high expression of CD56, CD16, DNAM-1 and NKG2D in the absence of CD3. Ex vivo-generated NK cells mediated efficient lysis of K562 and HL-60 target cells. Interestingly, these effectors showed a marked cytotoxic activity also against primary ALL and AML blasts (40% mean cytotoxicity at a 50:1 E.T ratio). *Conclusions.* These data indicate that clinical protocols for patients affected by acute leukemia based on the in vivo infusion of ex vivo GMP expanded NK cells appear feasible both in the autologous setting or after an allogeneic stem cell transplant, particularly considering that the infusion of NK cells should induce a very limited toxicity and no or a very low risk of graft-versus-host disease, thus avoiding the potential complications associated to the infusion of donor T-lymphocytes.

CO-024

DONOR-DERIVED WT-1 SPECIFIC CYTOTOXIC T CELLS DIRECTED AGAINST PATIENT'S LEUKAEMIA BLASTS FOR ADOPTIVE IMMUNOTHERAPY AFTER ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Introduction. The Wilms tumor antigen, WT1, has been described as a key molecule for tumor proliferation in a large number of human malignancies. Over expression of WT1 has been documented in various types of leukemia and solid tumors. In this study, we investigated the feasibility of *in vitro* generating and expanding WT1 peptide-specific T cells of donor origin and investigated their capacity to lyse patient's leukemia blasts (LB), expressing WT1, as a prerequisite for adoptive T-cell therapy after allogeneic hematopoietic stem cell transplantation (HSCT). *Methods.* Using a slightly modified methodology, previously employed for the induction of anti-leukemia CTLs directed against whole tumor cells (LB-CTLs), we evaluated the possibility of generating WT1-specific CTLs in 5 donor/recipients pairs starting from peripheral blood (PB) of HLA-A2+ HSC donors. CTLs were generated starting either from PBMC or CD8-enriched lymphocytes stimulated with DC pulsed with WT1 peptides in the presence of IL-7 and IL-12. Cells were restimulated in the presence of irradiated donor mononuclear cells pulsed with WT1-peptides and expanded in an antigen independent way. *Results*. CTLs displayed high levels of cytotoxicity against WT1-pulsed donor PHA-blasts, media 50% +/- 8% at effector/target (E:T) 25:1, and negligible levels (<10% lysis at E:T ratio of 25:1) against donor PHA-blasts pulsed with irrelevant peptides or with medium alone. WT1-specific CTLs from 4 out 5 donors displayed sizeable levels of cytotoxicity against patients' LB (media 37% +/- 13% at E:T ratio of 25:1). Patients' LB that were not lysed by donor-derived WT1-specific CTLs expressed lower levels of WT1 compared to other primary blasts. *Conclusions*. These data confirm the possibility of obtaining a large quantity of donor-derived WT1-specific CTLs, able to lyse patients' LB, from PB of HSCT donors, and suggest the possibility of utilizing these in adoptive immunotherapy to control/prevent leukemia relapse in HLA-A2 patients given allogeneic HSCT. WT1-specific CTL infusions could represent an alternative approach in case of an insufficient number of primary LB to generate LB-CTLs, or be used together with LB-CTLs, directed against the whole leukemia cells, to improve their efficacy. Further experiments are in progress to evaluate the possibility of generating WT1-specific CTLs from more donor/recipient pairs and their phenotypical and functional characteristics and compare levels of lysis against LB and WT1 expression levels on leukemia samples.

Physiopathology of Hemopoiesis

CO-025

EFFICACY OF THALIDOMIDE IN THE TREATMENT OF SEVERE RECURRENT EPISTAXIS IN HEREDITARY HEMORRHAGIC TELANGIECTASIA

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Introduction. Hereditary hemorrhagic telangiectasia (HHT; OMIM 187300 and 600376), also known as Rendu-Osler-Weber syndrome, is an autosomal dominant disease that leads to multiregional angiodysplasia. Recurrent and severe epistaxis, due to the presence of telangiectasias in nasal mucosa, is the most common presentation of HHT, frequently leading to severe anemia requiring intravenous iron and blood transfusions. In the management of HHT epistaxis, multiple approaches, including surgical options, have been tried, but all approaches are largely palliative with variable results, many requiring repeated interventions. Recently, angiogenesis has been implicated in the pathogenesis of HHT; therefore, anti-angiogenic substances may be effective in the treatment of vascular malformations in this disease. The aim of our prospective, non-randomized study was to assess the clinical effects of thalidomide therapy on the severity of epistaxis in patients with HHT refractory to standard therapy. *Methods*. HHT patients with at least one episode of overt bleeding/week requiring at least one blood transfusion during the last three months and refractory to mini-invasive surgical procedures were enrolled. Thalidomide was administered at a starting dose of 50 mg/day orally. In the event of unsatisfactory/no response thalidomide dosage was increased by 50 mg/day every 4 weeks until complete or partial response, to a maximum dose of 200 mg/day. Monthly follow-up was based on the epistaxis severity score and transfusion need, with adverse events being reported. The study is currently recruiting participants. *Results*. Eight patients, 6 M and 2 F, aged 51-80 years (median 67), have been enrolled so far and 4 have completed at least 16 weeks of treatment. Six out of 7 evaluable patients responded within 4 weeks of starting the drug: cessation of nose bleeding was observed in one case, reduction in the severity of epistaxis in 5 cases. One patient achieved partial response after 8 weeks of treatment. Thalidomide therapy significantly decreased also the need for red blood cell transfusions and improved the quality of life. Only nonserious adverse effects were observed during treatment, including constipation and drowsiness. No major side effects were reported. In no patient thalidomide had to be discontinued. *Conclusions*. These preliminary results indicate that thalidomide, with its antiangiogenic mechanism of action, may be an effective agent for the treatment of epistaxis in HHT patients unable to benefit from other available modalities of treatment.

CO-026

INTEGRIN ALPHA E (CD103) EXPRESSION MARKS A SUBSET OF HUMAN CD34+ CELL -DERIVED LANGERIN+ DENDRITIC CELLS (DCS) WHICH INDUCE T REGULATORY CELLS (TREGS) VIA INDOLEAMINE 2,3-DIOXYGENASE (IDO)

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Introduction. IDO is the rate-limiting enzyme in tryptophan catabolism along the kynurenine pathway.IDO expressed by different cell subsets inhibits T cell activation, proliferation and survival and induces Tregs,thus mediating immunological tolerance. Although human monocyte-derived DCs have been shown to express IDO, little is known about its expression in other subsets. *Methods*. CD34+-derived DCs were generated from healthy donors from purified CD34+ cells after 7 days of culture with GM-CSF, TNF- and Flt-3. Then, DCs were separated into CD1a-CD14+ and CD1a+CD14- cells. DCs subsets were analyzed for IDO expression by real-time PCR, western immunoblot, immunofluorescence and immunohistochemistry.The IDO enzyme activity was analyzed by kynurenine production and its function by inhibition of allogeneic proliferation and Tregs induction. *Results*. CD34+ cells did not express IDO mRNA regardless of the progenitor cell source. During DC differentiation, IDO expression and function, evaluated by enzymatic and immunological tests, was markedly induced at day 7. Interestingly, the expression of IDO was shown to be 10 times higher in the CD1a+ compartment as compared to CD1a- cell fraction. IDO expression resulted in increased production of kynurenine and in reduced allostimulatory capacity of T cell proliferation. Moreover, CD1a+ cells were shown to induce a population of CD4+CD25+Foxp3+ which acted as Tregs by inhibiting allogeneic T cell proliferation. This effect was abrogated by the addition of IDO inhibitor 1-methyl tryptophan. Phenotypically, IDO-expressing CD1a+ cells expressed CD207 and CD103, which has been recently identified as a marker for tolerogenic DCs. Importantly, IDO expression was mainly detected in the CD103+ CD207+ fraction, which induces Tregs through an IDO-dependent manner. Accordingly, CD103+ cells sorted at day 7 from CD34+ cell culture induce Tregs through an IDO-dependent mechanism and inhibited T cell alloproliferation. In vivo, immunohistochemical analysis allowed the detection of IDO-expressing CD1a+CD14- cells in the dermis of human skin biopsies. *Conclusions*. DC differentiation from CD34+ cells results in the expression of a functionally active IDO protein in CD103-expressing Langerin+DCs, which can be detected in vivo. Given the role of IDO in immune tolerance, a subset of bone marrow-derived DCs, expressing CD103, may be intrinsically committed to function as regulatory DCs.

CO-027

MODULATION OF GAMMA GLOBIN GENES EXPRESSION BY A NEW HISTONE DEACETYLASE INHIBITOR

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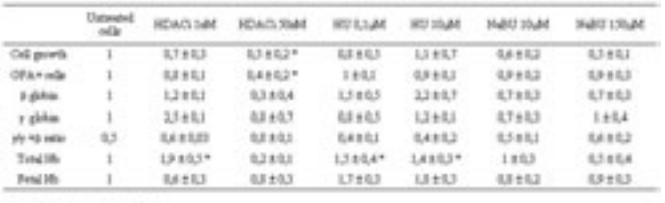
Introduction. Induction of fetal hemoglobin (HbF) is a promising therapeutic approach for the treatment of -thalassemia and sickle cell anemia (SCA). Several pharmacological agents, such as hydroxyurea (HU) and butyrates, have been shown to induce -globin genes. However, their therapeutic use is limited due to a relatively weak and variable efficacy, a cytotoxic and inhibitory effect on erythroid differentiation and to possible long-term side effects. At present hydroxyurea is approved for treatment of SCA, while in non transfusion-dependent -thalassemias it shows variable and moderate effects. Thus, more effective agents that can induce hemoglobin (Hb) with low toxicity are needed. The histone deacetylase inhibitors are potential therapeutic Hb inducers able to modulate gene expression through decondensation of chromatin structure. *Aims.* To investigate the effects of a new histone deacetylase inhibitor (HDACi) on erythropoiesis and hemoglobin synthesis. *Methods.* An *in vitro* model of erythropoiesis derived from human peripheral CD34+ cells from healthy volunteers was used. Effects of HDACi on erythroid proliferation and differentiation and on Hb synthesis were investigated and compared to hydroxyurea (HU) and butyrate (NaBU) efficacy. HDACi (concentration from 1 to 250 nM) was added every two days to cell cultures and Hb synthesis was evaluated by real-time PCR and ELISA assays. *Results.* HDACi affected erythroid differentiation and Hb synthesis in a dose-dependent manner. At high concentrations (i.e.50nM) the drug reduced cell proliferation compared to untreated cells, delayed erythroid differentiation (decrease of Glycophorin A+ [GPA+] cells) and increased the / + globin gene ratio but not the Hb levels (Table 1).

Table 1.



The increase in globin genes expression however could be related to the inhibitory effect on erythroid differentiation, thus to the presence of

an higher percentage of immature erythroblasts physiologically expressing higher levels of HbF, rather than to a specific effect of the drug on -gene transcription. HDACi at lower concentrations (i.e.1nM) positively affected hemoglobin production with an increase in and globin gene expression and in total hemoglobin (HbT) synthesis, with no effects on cells proliferation and differentiation. The HDACi 1nM efficacy in Hb induction was similar to that of hydroxyurea and higher than butyrate. *Conclusions.* HDACi at low concentration (1nM) increases Hb synthesis without affecting cell viability and differentiation. It increases the expression of both and globin genes, positively regulating the production of total hemoglobin and not only the fetal one (HbF). HDACi 1nM efficacy in Hb induction is similar to hydroxyurea and is better than butyrate. These findings support the evaluation of HDACi, used at low concentration, as a new candidate molecule for hemoglobinophaties treatment, in particular for SCA, due to its positive effect on -globin transcription and total hemoglobin production.

CO-028

TISSUE INHIBITOR OF METALLOPROTEINASES-1 (TIMP-1) MODULATES THE PROLIFERATION OF BOTH HUMAN CD34+ HEMATOPOIETIC STEM/PROGENITOR CELLS AND ACUTE MYELOID LEUKEMIA CELLS

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Introduction. Hematopoietic Stem Cells (HSCs) reside within the bone marrow (BM) stem cell niche, a nurturing environment long believed to protect HSCs from external insults. Nonetheless, recent findings showed that HSCs can respond to pro-inflammatory cytokines such as interferons or TNF , previously believed to modulate the function of immune cells only. The chronic activation of inflammatory circuits has also been shown to play a role in the onset of hematopoietic tumors, such as multiple myeloma and leukemias. Beside its role in tissue remodelling (as an inhibitor of Metalloproteinases), the Tissue Inhibitor of Metalloproteinases-1 (TIMP-1) also displays cytokine-like functions and its expression is dramatically increased in response to pro-inflammatory cytokines, making TIMP-1 a key player in flogosis. We previously found that TIMP-1(-/-) mice have decreased BM cellularity and that the engraftment capability of TIMP-1(-/-) HSCs is impaired, due to cell-cycle dysregulation. Here, we investigate the role of TIMP-1 on the proliferation of both normal human hematopoietic stem cells (HSCs) and acute myeloblastic leukemia (AML) cells. *Methods.* We measured TIMP-1 serum and plasma levels in the peripheral blood (PB) and the BM of healthy donors and compared them to those of patients with AML (both primary and secondary AML). Subsequently, we assessed TIMP-1 capability of modulating both HSC and leukemic cell proliferation by *in vitro* liquid cultures and colony-forming assays (CFU-C). The effects of TIMP-1 on more immature HSCs was also evaluated by *in vitro* Long-Term Cultures (LTCs), whereas primitive stem cell potential was assessed by transplantation of TIMP-1-treated CD34+ cells into NOD/Shi-scid/IL-2R null mice. *Results*. We found that TIMP-1 stimulates *in vitro* proliferation of both PB-mobilized and BM-derived CD34+ HSCs (in a dose-dependent manner), as shown by liquid cultures and clonogenic assays. Similar results were observed in AML blasts, with TIMP-1 promoting AML proliferation in primary leukemia CFU-Cs, even at the lowest tested concetrations. LTC assays showed that TIMP-1 also increases the number of LTC-Initiating Cells (LTC-ICs). However, the engraftment potential of normal CD34+ HSCs appeared to be decreased upon treatment with TIMP-1, as suggested by preliminary transplantation assays into immunodeficient mice. *Conclusions*. Our findings suggest that TIMP-1 may be a regulator of cell proliferation in both human hematopoietic stem/progenitors and AML cells and may provide new insights on how acute or chronic inflammatory conditions can affect both physiologic hematopoiesis or leukemogenesis. As such, TIMP-1 may represent a new target of therapies aiming at downregulating inflammatory responses in the BM microenvironment.

CO-029

HUMAN HEMATOPOIETIC STEM CELLS IN COCULTURE WITH OSTEOBLASTS: MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF THE HEMATOPOIETIC STEM CELL NICHE

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Introduction. Hematopoietic stem cells (HSCs) are located in the bone marrow (BM) in a specific microenvironment referred as the hematopoietic stem cell niche. In this specific microenvironment, HSCs interact with a variety of stromal cells including fibroblasts, endothelial cells, mesenchymal stem cells, reticular cells, osteoblasts and adipocytes. Though several components of the stem cell niche have been identified, the regulatory mechanisms through which such components regulate the stem cell fate are still unknown. In order to address this issue, we investigated how osteoblasts (OBs) can affect the molecular and functional phenotype of HSCs and vice versa in a coculture system. *Methods*. We set up a coculture system composed of human CD34+ cells in culture with human OBs purified from the trabecular bone. After coculture, CD34+ cells and the hematopoietic cell fraction were separated from OBs and analyzed by gene expression profiling, clonogenic assay and long-term culture to assess how OBs could affect the self-renewal and differentiation capacity of HSCs. *Results*. Our results showed that coculture with OBs induces a strong increase in the clonogenic capacity of CD34+ cells and a five-fold expansion of the CD34+CD38- progenitors pool. Moreover, clonogenic assay results showed an increase of the macrophage colonies coupled to a decrease of the erythroid ones. In the long-term culture, OBs seem to favour the differentiation of hematopoietic cells towards the monocytic lineage at the expense of the granulocytic and erythroid ones. Gene expression profiling allowed us to study which signalling pathways were activated in the hematopoietic cell fraction and in OBs after coculture. Such analysis enabled us to identify several cytokine-receptor networks, such as WNT signaling pathway, and transcription factors, as FOXC1 and TWIST, that could be activated by coculture with the stromal cell compartment and could be responsible for the biological effects reported above. *Conclusions*. Our results show that OBs are able to increase the clonogenic capacity of CD34+ cells and are capable of maintaining the more primitive hematopoietic progenitors up to 2 weeks in culture. Moreover, OBs are able to affect the differentiation capacity of CD34+ cells by favouring mono/macrophage commitment at the expense of the erythroid and granulocytic lineages. Gene expression profiling allowed us to identify new candidate transcriptions factors and signalling pathways underlying the effect exerted by OBs on HSCs. A better understanding of how the niche participates in the maintenance of hematopoiesis offers new opportunities for the development of novel HSCs ex-vivo expansion protocols that allow stem cells expansion without loss of 'stemness'.

CO-030

THE ALTERED BALANCE BETWEEN THE BCL-2 FAMILY PROTEINS BCL-XL AND BAK CONTRIBUTES TO THE INCREASED PLATELET CLEARANCE IN IMMUNE THROMBOCYTOPENIA

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Introduction. Based on a mouse model, it has recently been shown that an intrinsic program for apoptosis controls platelet survival and dictates their life-span. Specifically, pro-survival Bcl-XL counteracts the pro-apoptotic activity of BAK to maintain platelet survival, but as Bcl-XL degrades, aged platelets are primed for death. Bcl-XL deficiency does not impair platelet production. By contrast, genetic ablation or pharmacological inactivation of Bcl-XL reduces platelet half-life and causes thrombocytopenia in a dose-dependent manner. Deletion of BAK corrects these defects. Thus, platelets are genetically programmed to die by apop-

tosis and the antagonistic balance between Bcl-XL and BAK constitutes a molecular clock that determines platelet life-span (Cell 2007). Interestingly, this mechanism of regulation of survival has never been characterized in platelets from patients with Immune Thrombocytopenia (ITP). Therefore, in the present study, we investigated whether in ITP abnormalities of the Bcl-XL/BAK system may play a pathogenetic role. Methods. For these purposes, apoptosis together with the expression of Bcl-XL and BAK molecules have been characterized on freshly isolated and *in vitro* aged platelets of healthy subjects and ITP patients by flow cytometry. Platelets were incubated at 37°C for different time points (24, 48, 72, 168 hours) in autologous platelet poor plasma. After platelet labelling with specific monoclonal antibodies, the percentage of positive cells has been evaluated at flow cytometry. We studied 8 patients with active ITP: 3 patients were newly diagnosed and 5 patients had chronic ITP. The median number of platelets was 42.000/mm^3 (range 24.000-69.000). *Results*. We found that: 1) aged platelets from ITP patients show increased apoptosis as compared with the normal counterparts; 2) the mean percentage of freshly isolated platelets expressing BAK is significantly higher than that of platelets expressing Bcl-XL, both in controls (4.55+/-3.0% vs 1.80+/-0.7%; P<0.05) and ITP patients (5.15+/-3.12% vs 2.27+/-1.4%; P<0.05); 3) the mean percentage of freshly isolated platelets expressing Bcl-XL and BAK is comparable between patients and controls; 4) the mean percentage of aged platelets from ITP patients expressing Bcl-XL is significantly reduced as compared with that of healthy controls after 48 (0.79+/-0.80% vs 3.48+/-1.10%; P<0.03) and 72 (0.20+/-0.18% vs 1.24+/-0.17%; P<0.02) hours of incubation, respectively; 5) the mean percentage of aged platelets from ITP patients expressing BAK is comparable with that of healthy controls after 24 (11.64+/-4.13% vs 11.85+/-1.67%), 48 (7.60+/-5.88% vs 8.71+/-4.51%), 72 (2.28+/-0.60% vs 4.38+/-3.55%) and 168 (3.59+/-0.93% vs 3.17+/-1.93%) hours of incubation, respectively. *Conclusions*. Our results therefore demonstrate that in ITP the balance between Bcl-XL and BAK is altered and suggest that this finding may play a pathogenetic role in the accelerated platelet clearance.

CO-031

HUMAN DENDRITIC CELLS INDUCE TREGS THROUGH THE PGE2-INDEPENDENT EXPRESSION OF AN ACTIVE FORM OF IDO2 ENZYME

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Introduction. As indoleamine 2,3-dioxygenase (IDO1), indoleamine 2,3-dioxygenase-like (IDO2) catalyzes tryptophan degradation into kynurenine and is inhibited by 1-methyl-tryptophan (1-MT). Inflammatory mediators, such as PGE2, induce IDO1 expression, which, in turn, enhances tolerogenic features of dendritic cells (DCs) when it is not degraded through SOCS3. Conversely, the regulation of IDO2 expression and function in human DCs is under investigation. *Methods.* Circulating myeloid (CD1c+), plasmacytoid (CD303+) DCs and CD14+ were immunomagnetically purified. Monocyte-derived DCs (moDCs) were obtained from CD14+ cultured with GM-CFS and IL-4. Circulating DCs and moDCs were matured with IL-1 , IL-6, TNF- with or without PGE2. IDO1 and IDO2 expression were evaluated by qPCR and Western Immunoblot before and after SOCS3 immunoprecipitation. IDO1 and IDO2 function was evaluated by kynurenine production and Tregs induction with or without IDO1 and IDO2 inhibition. *Results*. Human circulating myeloid DCs express a higher level of IDO2 mRNA in comparison to circulating plasmacytoid DCs. Circulating DCs generate Tregs thorough IDO2, since the addition of its specific inhibitor 1-MT-D represses Tregs generation. To evaluate IDO2 regulation and function, moDC were used as experimental model. IDO2 gene expression is upregulated by PGE2, whereas the protein expression is independent of PGE2. Moreover, when gene transcription or protein translation are inhibited, IDO2 protein expression is persistent both in immature and in mature (with or without PGE2) DCs. SOCS3 immunoprecipitation and proteasomal inhibition show that SOCS3 does not bind IDO2 which is not, therefore, driven to proteasomal degradation. The silencing of IDO2, through specific siRNA, downregulates kynurenine production and Tregs generation. Interestingly, in circulating DCs, PGE2 upregulates both IDO1 and IDO2 gene expression in myeloid, but not in plasmacytoid subset. *Conclusions.* These data demonstrate that, besides IDO1, human DCs express also IDO2. Unlike IDO1, IDO2 expression is independent of inflammatory mediators, such as PGE2, and does not require continuous synthesis, being stably expressed. Like IDO1, IDO2 expression contributes to tolerance in human DCs because it is functionally active, by degrading tryptophan into kynurenine and by inducing Tregs. Therefore, in DCs, IDO1 expression enhances their tolerogenic functions under inflammatory conditions, while IDO2 expression contributes to their tolerogenic features in steady-state conditions.

CO-032

VALPROIC ACID FAVOURS ERYTHROID AND MEGAKARYOCYTE DIFFERENTIATION OF HUMAN CD34+ CELLS INDUCING GFI1B AND MLLT3 EXPRESSION

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Introduction. Chromatin modifications such as histone acetylation and DNA methylation are important mechanisms of gene expression control. More in detail, specific histone acetylation and DNA methylation patterns are known to be associated with modification of chromatin structure and transcriptional regulation. Histone deacetylation and DNA methylation of promoters have been correlated to the negative regulation of gene expression, while inhibition of both histone deacetylation and DNA methylation is able to revert the silencing effect. Given the aberrant expression and/or inappropriate activation of histone deacetylases (HDACs) and DNA methyltransferases (DMTs) in tumors, compounds targeting such enzymes have recently generated great interest as anticancer drugs. Histone deacetylase (HDAC) inhibitors are widely used in therapy for hematological malignancies; nevertheless, little is currently known concerning their effects on normal myelopoiesis. *Methods*. In order to investigate the effect of HDAC inhibitors on the myeloid commitment of hematopoietic stem/progenitor cells (HSPCs) we treated CD34+ cells with valproic acid (VPA). Moreover, we investigate changes in gene expression profile induced by VPA treatment on HSPCs, by means of microarray analysis. *Results*. Our results demonstrated that VPA treatment induces H4 histone acetylation in CD34+ cells and blocks them in the G0-G1 phase of cell cycle. CD34 protein expression was maintained at higher levels in VPA treated cells, while its physiological downregulation occurred in control cells. Moreover, VPA treatment enhanced erythrocyte and megakaryocyte differentiation at the expense of the granulocyte and mono-macrophage lineages, as demonstrated by immunophenotypic, morphological and clonogenic analysis. Finally, we demonstrated that VPA up-regulated master regulatory genes of the erythrocyte and megakaryocyte differentiation (GFI1B and MLLT3) through histone hyper-acetylation at their promoter sites. *Conclusions.* Our results showed that VPA induces block of proliferation coupled to increased expression of stemness-related markers in HSPCs, suggesting that VPA treatment could lead to expansion of the stem cell compartment *in vitro*. Moreover, our data indicate that VPA treatment affects lineage choice by enhancing erythrocyte and megakaryocyte differentiation at the expense of granulocyte and mono-macrophage lineages. These results provided for the first time an important molecular support to explain the biological effect of VPA treatment on proliferation, self-renewal and differentiation in human HSPCs and allowed to improve knowledge on activity of antineoplastic drug VPA on normal hematopoiesis.

Chronic Lymphocytic Leukemia 1

CO-033

SLAMF1/CD150 IS A SIGNALING RECEPTOR EXPRESSED BY A SUBSET OF CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS CHARACTERIZED BY A FAVORABLE PROGNOSIS

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Introduction. Human SLAMF1 (CD150) is the prototype member of a family of receptors expressed on the surface of hematopoietic cells. SLAMF-1 cognate binding initiates distinct signal transduction networks in T cells, natural killer cells and antigen presenting cells. Gene expression profiling has identified SLAMF1 as part of the genetic signature characterizing chronic lymphocytic leukemia (CLL) patients with favorable prognosis. The aim of this work is to validate the prognostic impact of SLAMF-1 in a cohort of CLL patients and to investigate its role in the activation of signaling pathways influencing CLL homeostasis. *Methods.* SLAMF-1 expression was tested in a cohort of 270 clinically and molecularly characterized CLL patients by flow cytometry. To start signaling, SLAMF-1 was ligated with an agonistic antibody and the ensuing cytoplasmic events determined by biochemical analyses. The autophagic pathway was studied using confocal and transmission electron microscopy. Apoptosis was determined by AV/PI assays. *Results.* The analysis of SLAMF1 surface expression on the CD19+ fraction of 270 CLL patients revealed highly variable levels (1-95%). Statistical analyses of the data indicate that patients characterized by a good prognosis (in terms of disease stage at diagnosis or treatment requirements) express higher levels of SLAMF1 compared to the counterpart. Moreover, patients with > 6% SLAMF1+/CD19+ CLL cells had a significantly longer treatment free survival (median 6.4 in SLAMF1+ vs 1.2 years in SLAMF1- patients, P=.002). Consistently, SLAMF1 expression was also inversely correlated with CD38 and CD49d, two molecular markers of unfavorable prognosis and positively associated with the presence of somatic mutations in the IgHV genes. Engagement of SLAMF1 by a specific mAb started a signaling cascade mediated by phosphorylation of the EAT-2 adaptor and downstream activation of Vav-1, p38 and JNK. Co-crosslinking of SLAMF1 with sIgM prolonged phosphorylation of p38 and JNK and increased the percentage of CLL cells undergoing apoptosis, as compared to either signal alone. Furthermore, activation of the SLAMF1 pathway for a period of 6 hours led to the increased appearance of autophagic vescicles, as confirmed by confocal and transmission electron microscopy. The relative modulation of apoptosis and autophagy was mediated by the sequential phosphorylation of JNK and Bcl-2: the end result is the activation of Bcl-2 and the release of beclin-1, an essential member of the autophagic complex. *Conclusions.* In conclusion, SLAMF-1 represents a novel marker for the subset of CLL patients characterized by an indolent clinical course. Functional data suggest that it may function together with the BCR in regulating CLL apoptosis and that it can keep the autophagic process active, maintaining homeostasis of CLL cells. These findings suggest a hypothetical link between the relative modulation of autophagy and apoptosis and a more favorable clinical outcome in CLL.

CO-034

THE PD-1/PD-L1 AXIS CONTRIBUTES TO T CELL DYSFUNCTION IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction. Chronic Lymphocytic Leukemia (CLL) is marked by profound defects in T-cell function. Programmed death-1 (PD-1) is a cell surface molecule that inhibits activation and is involved in tumor escape mechanisms through binding of the specific PD-L1 ligand. *Methods.* We compared T cell subpopulations of CLL patients (n=120) to age- and sex-matched healthy donors (HD, n=30) using multiparameter flow cytometry. Immunohistochemical analyses were used to study PD-1 and PD-L1 expression in the lymph node microenvironment. Functional assays were used to determine the involvement of the PD-1/PD-L1 axis in shaping T cell responses. *Results*. The first finding of this work is that CD4+ T lymphocytes from CLL patients express significantly higher levels of the PD-1 receptor, as compared to the same cells purified from age- and sex-matched donors (52% vs 34%, P<.001). In keeping with the notion that PD-1 is a marker of cell exhaustion, we found that CD4+ T lymphocytes from CLL patients display increased numbers of effector memory cells with a concomitant decrease in naïve and central memory cells, when compared to age- and sex-matched donors. As expected, the number of effector memory cells positively associated with a more advanced stage of disease, treatment requirements and unfavorable genetic aberrations. On the other side, leukemic lymphocytes expressed higher levels of PD-L1 than circulating B lymphocytes from normal donors. PD-1 and PD-L1 increased dramatically when T or B lymphocytes were treated with mitogenic signals (e.g., PHA or PMA, respectively), suggesting that this interaction might work efficiently in an activated environment. This hypothesis was tested by determining PD-1 and PD-L1 expression in the proliferation centers located in the lymph nodes of CLL patients. Results indicate that PD-L1+ proliferating CLL cells are in close contact with CD4+/PD-1+ T lymphocytes. Lastly, functional experiments performed using anti-PD-1 antibodies or recombinant PD-L1 ligands clearly indicate that this axis contributes to driving IL-4 secretion and to the inhibition of IFN-gamma production by CD8+ T cells. *Conclusions.* CD4+ T lymphocytes from CLL patients express high levels of the surface marker PD-1 and exhibit an exhausted phenotype, while CLL cells express the PD-L1 ligand. Functional data suggest that the PD-1/PD-L1 interactions are critical in skewing the T cell compartment towards a Th2 phenotype, by impairing IFN-gamma secretion by CD8+ cells. These observations imply that pharmacological manipulation of the PD-1/PD-L1 axis might be relevant in restoring T cell functions.

CO-035

NOTCH1 AND SF3B1 MUTATIONS IN CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) PATIENTS REQUIRING FIRST-LINE TREATMENT: CORRELATION WITH BIOLOGICAL PARAMETERS AND RESPONSE TO TREATMENT

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Introduction. The introduction of whole exome sequencing has allowed to unravel novel molecular lesions in CLL. Among others, NOTCH1 and SF3B1 mutations are overall detected in 4-12% and 5-17% of patients, respectively. In retrospective studies, their presence has been shown to correlate with overall survival (OS) and treatment-free interval shortening. *Methods*. To define the incidence and clinical impact of

NOTCH1 and SF3B1 mutations in patients requiring first-line treatment, we evaluated 162 CLL patients enrolled in the GIMEMA LLC0405 protocol (n=80) for patients aged <60 yrs, or in the ML21445 protocol (n=82), for elderly patients (aged >65 yrs or 60-65 if not eligible for fludarabine), respectively. In the GIMEMA LLC0405, patients were stratified into low and high-risk: patients with del17p or with del11q plus an unmutated IGHV status and/or CD38 positivity and/or ZAP70 positivity were considered as high-risk and underwent Fludarabine plus Campath, followed by stem cell transplantation procedures, whereas low-risk patients received Fludarabine and Cyclophosphamide. The MLL21445 protocol consisted of 8 cycles of Chlorambucil and 6 of Rituximab as induction. NOTCH1 exon 34 and SF3B1 exons 14 and 15 were screened by Sanger sequencing on material collected at the time of treatment. *Results.* NOTCH1 mutations were detected in 18 (22%) cases enrolled in the LLC0405 study. There was a significant association with high-risk stratification (P=0.036), an IGHV unmutated status (P=0.0035), CD38 (P=0.03) and, partly, ZAP70 expression (P=0.059), and with +12 (P=0.034). The complete response (CR) rate was lower in NOTCH1 mutated patients (P=0.052), whereas so far no significant difference between mutated and wild-type patients has emerged in terms of OS and progression-free survival (PFS). SF3B1 mutations were recorded in 9 (11%) cases and no significant associations were found with known biological parameters and response to treatment. In the ML21445 cohort, NOTCH1 mutations were found in 12 (15%) cases, were associated with an unmutated IGHV status (P=0.047) and ZAP70 expression (P=0.007) and did not impact on the CR rate. SF3B1 mutations were detected in 11 cases (13%) and were rarely detected in IGVH mutated cases; no significant associations were found with known biological parameters. Interestingly, among SF3B1 mutated patients, only 1 (11%) achieved a CR. NOTCH1 and SF3B1 mutations were mostly mutually exclusive among each other and with TP53 lesions in the whole cohort. *Conclusions*. This study confirms the association of NOTCH1 mutations with unfavorable biologic markers and +12, while the presence of SF3B1 mutations was not coupled to poor prognostic markers in patients requiring first-line treatment. Furthermore, it suggests that NOTCH1 mutations impact on the CR rate of patients receiving Fluda-based regimens, while SF3B1 impacts on the CR rate of patients treated with Chlorambucil and Rituximab.

CO-036

THE PI3-KINASE INHIBITOR, GS-1101 (CAL-101), INHIBITS CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) CELL SURVIVAL AND ADHESION IN ENDOTHELIAL AND MARROW STROMAL CELL CO-CULTURES

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Introduction. CLL cells display constitutive PI3K pathway activation, presumable due to CLL interactions with the microenvironment. GS-1101 is a potent and selective inhibitor of the PI3K delta isoform and has shown promising clinical activity in chronic lymphocytic leukemia (CLL) in on going clinical trials. We investigated the ability of GS-1101 to disrupt interactions between CLL and endothelial cells (EC) or bone marrow stromal cells (BMSC). *Methods.* The viabilities of CLL cells in co-culture with two different EC lines (HUVEC and UV-2) and two different BMSC lines (KUSA-H1 and NKtert) were determined by staining with DIOC6 and PI. Adhesion of CLL cells to EC (HUVEC and HMEC-1) and BMSC (CLL-MSC and 9-15c) was evaluated by two different methods: flow cytometry and fluid shear stress assay. In both cases ECs and BMSCs were stimulated with TNF (10ng/ml) for 24h. *Results.* To investigate the role of ECs and BMSCs in supporting CLL viability, we cocultured CLL cells for 72h in presence or absence of EC or BMSC. We found that both, EC and BMSC rescue CLL cells from spontaneous apoptosis with significantly higher CLL cell viabilities in the presence of EC and BMSC. Both ECs and BMSCs rescue CLL cells from spontaneous apoptosis: BMSCs were more effective which may explain why the marrow is a preferred site for residual disease and relapse in patients with CLL. To test the effects of GS-1101 on EC- and BMSC-mediated CLL cell protection, CLL cells were cultured on ECs or BMSCs in presence or absence of 5 M GS-1101 and CLL cell viabilities were assessed at 24h, 48h and 72h (n=7). In a representative case, GS-1101 reduced CLL cell viabilities from 51.7% to 41.9% with HUVEC, 47.9% to 41% with UV-2, 97.5% to 64.9% with NKtert and 92.9% to 85.1% with KUSA-H1 at 48h. In bone marrow and secondary lymphoid tissues CLL cells interact and adhere to stromal cells and matrix. To investigate if GS-1101 affects CLL cells adhesion to ECs and BMSCs, we performed adhesion assays activating both ECs and BMSCs with TNF alpha to upregulate VCAM-1 expression. We observed that treatment with TNF significantly increases the ability of CLL cells to adhere to ECs and BMSCs and is reduced in presence of 5 M GS-1101 (Figure 1, n=8, *P< 0.05; **P< 0.01). We used a parallel plate flow assay in order to investigate if GS-1101 interferes with the dynamic interactions between CLL cells and EC (HUVEC). We observed that TNF alpha treatment significantly increases CLL cells adhesion to HUVEC compared with the unstimulated condition, while GS-1101 is able to reduce adhesion at low shear force of 15 dynes/cm2 and high shear force of 30 dynes/cm2. *Conclusions.* Our findings demonstrate a dual mechanism of decreasing microenvironmental pro-survival signals and reducing interactions that retain CLL cells in the tissue microenvironment. These effects are consistent with evidence of clinical activity as measured by a decrease in lymphadenopathy.

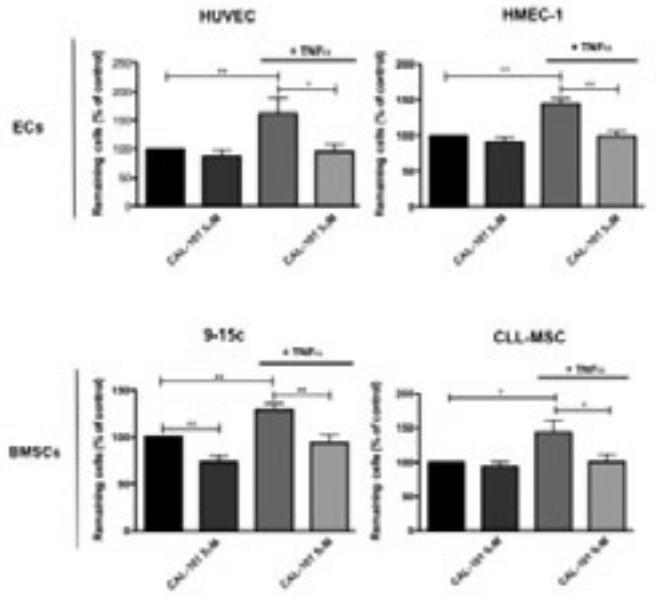

Figure 1.

CO-037

TP53 CODON 72 POLYMORPHISM IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA (CLL): CORRELATION WITH TP53 MUTATIONS, GENOMIC AND FUNCTIONAL FEATURES

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Introduction. A common single nucleotide polymorphism at codon 72 of TP53 gene (SNP72) results in a G/C variation, leading to a single aminoacidic change (CGC-arginine, CCC-proline). These 2 genotypes appear to be biochemically and biologically different in apoptosis induction. In the present study, we evaluated if the polymorphic variants of TP53 could influence the biology of chronic lymphocytic leukemia (CLL) cells. *Methods*. TP53 SNP72 was analyzed in 167 CLL cases by sequencing and was correlated to TP53 mutations (exons 4-9). To examine the effects of SNP72 in TP53 wild-type CLL, 36 cases underwent gene expression profile (GEP) using the HGU133 Plus 2.0 Affymetrix arrays and 36 CLLs were evaluated by Annexin V assay

after exposure to ionizing radiation (IR). *Results*. The frequency of TP53 genotypes (Arg72Arg 59% (n=98), Arg72Pro 33% (n=56), Pro72Pro 8% (n=13) among CLL cases was comparable with the published frequency in Caucasians. We found a significant correlation with the presence of TP53 mutations: Arg72Arg and Arg72Pro CLL displayed mutated TP53 in 21% and 11% of cases, respectively, while Pro72Pro CLL had TP53 mutations in 46% (P=0.014). Of 167 cases, 36 were evaluated for TP53 mutations at 2 consecutive time-points (median time interval: 43 months, range 7-147): none of 16 Arg72Arg CLL, 2/14 Arg72Pro and 3/6 Pro72Pro acquired TP53 mutations over time (P=0.001). A distinctive GEP was observed when 21 TP53 wild-type Arg72Arg cases were compared with 15 Arg72Pro/Pro72Pro CLLs. We found 51 differentially expressed genes involved in apoptosis regulation (PIM3, COL18A1, TRAF4, RB1CC1) (P=0.001), protein localization (BACE2, EZR, RNF103, LYST) (P=0.027) and immune response (BCL3, FOXP1, IL2RG) (P=0.035). When samples were subdivided according to their IGHV status, a common pattern of expression (167 genes) could be found for Arg72Pro/Pro72Pro cases only in the IGHV mutated group, including kinases (STK4, IRF3, MEKK) (P=0.009), protein regulating apoptosis (RB1CC1, CD44, COL18A1, CASP3, HSPA5) (P=0.021), cell motility (HIF1A, CORO1A, SP100) (P=0.010), transcription (MEF2C, PFN1, MED6) (P=0.021) and immune response (BLNK, CD79A, CD79B, CD44, IL27RA, FOXP1) genes (P=0.014). A functional assay, performed only on TP53 wild-type cases showed a significant increase in apoptosis upon IR only in 21 Arg72Arg CLL (P=0.002). Moreover, the amount of apoptotic cells after IR was significantly higher among 21 Arg72Arg vs 15 Arg72Pro/Pro72Pro (mean deltaApo: 18.8 +/- 12 vs 11.2 +/- 7.9; P=0.047). *Conclusions*. Pro72Pro CLL are highly associated with the presence and acquisition of TP53 mutations. Both heterozygous and homozygous proline variants exhibit a distinctive GEP involving apoptosis regulation and a relatively impaired apoptotic response to IR. TP53 SNP72 genotyping might allow an early recognition of patients who are likely to acquire TP53 mutations, and are less prone to apoptosis.

CO-038

DEVELOPMENT OF CHLORAMBUCIL/HYDROXYCHLOROQUINE-LOADED ANTI-CD20 NANOPARTICLES FOR THE TREATMENT OF CHRONIC LYMPHOCYTIC LEUKEMIA : *IN VITRO* MODEL

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Introduction. The actual challenge of tumor therapy is to design new therapeutic agents able to maximize the efficacy minimizing the adverse effects. Nanoparticles (NP) have emerged as important tools to modify the release profile for a large number of drugs. The aim of this study was to determine whether anti-CD20-coated biocompatible FDA-approved NP loaded with Chlorambucil (CLB) and Hydroxychloroquine (HCQ) could induce apoptosis of the B-Chronic Lymphocytic Leukemia (CLL) cell line MEC-1, as well as of circulating neoplastic B-cells isolated from patients with B-CLL. *Methods.* Nanoparticles were prepared by the copolymerization of polylactic acid and polycaprolactone and had an diameter of 250 nm. The NP with HCQ and CLB were prepared by encapsulation of the two drugs at concentrations of 165 µg per mg of polymer inside their core, and then modified by anti-CD20 adsorption. The NP were resuspended in PBS with 10% Bovine Serum Albumin (BSA) and maintained at 4°C before use. To investigate the ability of NP to affect cell viability and induce cellular apoptosis, MEC-1 cells (2 x 105) were incubated with anti-CD20 coated and CLB-HCQ loaded NP for 48 hours and the residual viable cells was determined with MTT assay. Cell death program activation was also evaluated by analyzing PARP-1 cleavage and Annexin V detection. Monoclonal B-cells were obtained from 44 patients affected by CLL. In each case the IGHV gene mutational status was assessed using standard methods and CD38 and ZAP-70 expressions were determined by flow cytometry. Interphase FISH was performed on nuclei preparations of PBMC and each case was investigated for 13q deletion, 11q deletion, 17p deletion or presence of trisomy 12. *Results.* Anti-CD20 NP with HCQ and CLB were able to induce a strong reduction (95%) in cell viability of MEC-1 cells. The cytotoxicity was present in a dose-dependent manner and was apoptosis-dependent as confirmed by the PARP-1 activation and by the binding of Annexin V on cell surface. The same high cytotoxicity rate (>90%) was found also in patient-derived B-cells. The killing rate was independent from IGVH mutational status or from ZAP70 and CD38 expression or from the presence of 17p deletion. An efficient cytotoxicity was present even in CLL B-lymphocytes expressing low CD20 antigen levels on cell surface. The effects of NP were also tested on CD20-negative human and animal cells (CHO, HUVEC, MEL-28, LoVo cell lines) and no specific cytotoxicity was obtained. On the contrary, incubation of CD20-negative cells with free HCQ + CLB induced cell killing between 58 and 71%. *Conclusions.* These results indicate that anti-CD20 coated and CLB-HCQ loaded NP show a high toxicity rate in CLL cell line as well as in B-cell obtained from CLL patients. The toxic effect is present also in 17p deleted cases, usually resistant to chemo-immuno-therapy. These results should be confirmed in animal models in order to introduce this promising technology into human clinical trials.

CO-039

CYTOGENETIC ABERRATIONS IN THE CD38 POSITIVE FRACTION OF CD38 NEGATIVE CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS: A MARKER OF AGGRESSIVENESS?

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Introduction. Chronic lymphocytic leukemia (CLL) is a disease with a high variability in clinical presentation and outcome. Even though many patients live for long periods with the disease, some cases may show a progression to a more aggressive leukemia which may be characterized by the acquisition of new genetic abnormalities either by clonal evolution or by an expansion of a clone with high risk aberrations. Prognostic parameters that correlate with worse clinical outcome include stage, the expression of CD38 and/or ZAP70, the unmutated configuration of the variable region of the immunoglobulin heavy chain gene (IGHV), the presence of specific chromosome aberrations and/or molecular mutations affecting TP53, NOTCH1, SF3B1 and BIRC3. However in patients with favourable prognostic features the biologic and molecular events leading to disease progression and the occurrence of new molecular cytogenetic lesions are largely unknown. We therefore studied the biologic and clinical significance of minor cytogenetically abnormal clones in the CD38 positive fraction of untreated CLL patients with good prognostic parameters (CD38 negativvity and normal karyotype or del(13)(q14) as single aberration). *Methods.* Twenty eight consecutive CD38 negative (Cd38 positive cells < 5%) CLL patients with normal karyotype or del(13)(q14) were evaluated in this study. CD38 positive and CD38 negative CLL cells were isolated by sequential immunomagnetic sorting (Dynabeads) following depletion of CD3, CD16, CD14 positive cells (purity > 99%). CD38 positive and negative cells were then analyzed by (i) FISH analysis using commercially available probes for the regions most frequently involved in CLL patients (13q14, 12q13, 11q22/ATM, 17p13/TP53, 14q32) and by (ii) micro-RNA expression. Data were then correlated with clinical parameters. *Results.* In 16/28 CD38 negative CLL patients, FISH analysis demonstrated the presence of minor (15-34% of the cells) cytogenetically abnormal clones within the CD38 positive fraction: 11q deletion in 7 cases, 17p deletion in 6, trisomy 12 in 5, 14q32 rearrangements in 1 case. According to FISH results patients were therefore classified as CLL with and without subclones in the CD38 positive fraction. By micro-RNA analysis we found that patients with subclones had a distinctive profile, when compared to patients without subclones, characterized by a downregulation of microRNA-125a-5p (a tumor suppressor in various malignancies) both in the CD38 negative and positive populations. With a median follow-up of 36 months, patients with subclones showed a higher need of treatment (9/16 cas-

es vs 1/11 in patients with and without subclones respectively, P=0.0159). Among CLL patients with subclones, 2 cases showed the emergence of a major clone in the peripheral blood sample (45-67% of the cells) with the same genetic lesions previously observed in the CD38 positive subpopulations. *Conclusions.* Our data showed that genetic instability within the CD38 positive fraction of CLL patients with favourable prognostic features (CD38 negativity and normal karyotype or del13q14) may favour the growth of small clones with poor prognosis cytogenetic aberrations which may be associated with microRNA-125a-5p disregulation, clonal expansion and, in some cases, disease progression.

CO-040

DIFFERENT IMPACT OF NOTCH1 AND SF3B1 MUTATIONS ON THE RISK OF CHRONIC LYMPHOCYTIC TRANSFORMATION TO RICHTER SYNDROME

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Introduction. Richter syndrome (RS) represents the development of an aggressive lymphoma, most commonly a diffuse large B-cell lymphoma (DLBCL), in the context of chronic lymphocytic leukemia (CLL). At least two types of RS exist: i) transformation of CLL into a clonally related DLBCL, that accounts for ~80% of cases; and ii) development of a DLBCL unrelated to the CLL clone. Biologically, clonally related RS frequently harbor genetic lesions of TP53 and MYC that are otherwise absent or exceptional in clonally unrelated RS. NOTCH1 and SF3B1 mutations are novel CLL genetic lesions associated with poor outcome. Here, we investigated 605 CLL to verify whether the poor prognosis predicted by NOTCH1 and SF3B1 mutations in CLL may be explained by an increased risk of developing RS.

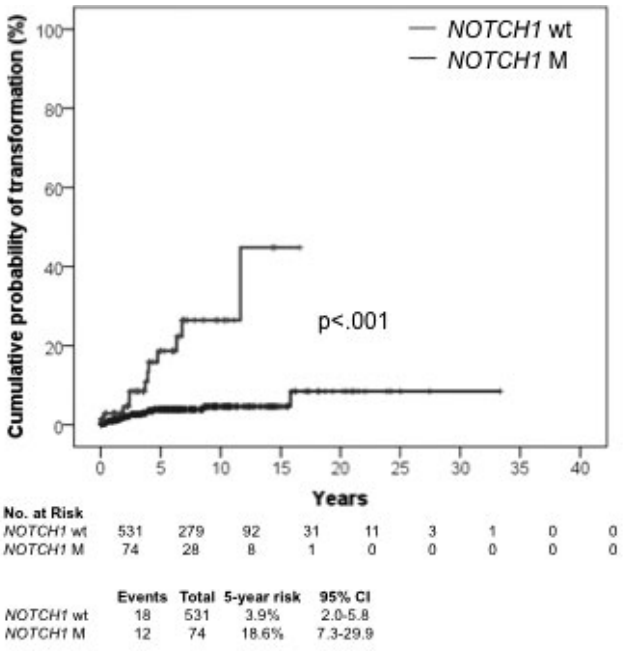

Figure 1.

Methods. NOTCH1 and SF3B1 mutations were analyzed by targeted Sanger resequencing. *Results.* At CLL presentation, NOTCH1 mutations occurred in (74/605) 12.2% patients, being mostly represented (82.4%) by the c.7544_7545delCT frameshift deletion. SF3B1 mutations occurred in (35/605) 5.8% cases and were mainly represented (50.0%) by the K700E missense substitution. After a median follow-up of 6.4 years, 30/605 (4.9%) CLL had transformed into a clonally related RS, while 10/605 (1.6%) had developed a clonally unrelated DLBCL. NOTCH1 mutations associated with a ~5.8 fold increase in the crude hazard of transformation into a clonally related RS (HR: 5.81; P<.001). Consistently, CLL harboring NOTCH1 mutations had a significantly higher cumulative probability of transforming into a clonally related RS (45.0% at 15 years) compared to CLL without NOTCH1 mutations (4.6% at 15 years) (P<.001) (Figure 1). Conversely, NOTCH1 mutations had no impact on the development of a clonally unrelated DLBCL (P=.494). NOTCH1 mutations were significantly enriched among cases harboring other factors known to affect RS risk, including CD38 expression (27.6%, P<.001) and IGHV4-39 gene usage (36.8%, P=.005), especially if rearranged in a stereotyped fashion in subset 8 (66.6%; P<.001). By multivariate analysis, NOTCH1 mutations were selected as an independent risk factor of transformation into a clonally related RS (HR: 3.71; P=.002), along with high risk cytogenetics (HR: 3.26; P=.002) and IGHV4-39 usage (HR: 3.14; P=.047). SF3B1 mutations showed no impact on RS development (P=.616). *Conclusions.* This study documents that NOTCH1 mutations, but not SF3B1 mutations, are an independent prognosticator of CLL transformation into clonally related RS, whereas have no impact on development of clonally unrelated RS. Importantly, up to 40-50% of NOTCH1 mutated patients are ultimately projected to develop and die of clonally RS. On these bases, the poor prognosis associated with NOTCH1 mutations in CLL may be explained, at least in part, by a substantial risk of developing RS.

Myelodysplastic Syndromes

CO-041

INTERPHASE FISH (I-FISH) REVEALS A PROGRESSIVE LOSS OF CHROMOSOME 7Q MATERIAL IN KARYOTYPICALLY NORMAL MDS PATIENTS DURING DISEASE OUTCOME

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Up to now no study carried out in chromosomally normal MDS patients has revealed the progressive loss of chromosome 7q material during disease follow-up and has estimated whether this phenomenon has any potential prognostic significance. The present study applied I-FISH in order to establish the prevalence of cryptic 7q-/-7 in 117 cytogenetically normal MDS patients and to evaluate whether in 7q- patients the increase of the deletion size could correlate with clinical evolution. I-FISH was performed with the commercial probes ON MDS 7q22/7q35 from Kreatech (Amsterdam, Netherland) and the RP11-95L16 and RP11-51M22 (located at 7q31 and covering the TES gene), the RP11-122A11 (7q34 region) and the RP11-992C19 (located at 7q36 and covering the EZH2 gene) BAC probes from BACPAC Resources Center at C.H.O.R.I. (Oakland, USA). All these BAC probes were labelled and applied as previously reported (Dambruoso et al, 2012 in press). I-FISH cut-off values calculated by applying a (one-sided) 95% confidence interval using a binomial distribution for the proportion of interphase cells with one and two red signals were fixed at 10%. Patients analysed were included within a previous series of 637 cases who came to our observation between January 2000 and December 2011; there were 51 females and 66 males with a median age of 65 years (range 28-83). According to WHO classification, 42 patients were classified as RA with and without ringed sideroblasts, 30 as RCMD with and without ringed sideroblasts, 3 as unclassifiable MDS, 24 as RAEB-1 and 18 as RAEB-2. Considering IPSS score, 44 patients were considered low-risk, 29 intermediate-1 risk and 30 intermediate-2 risk and 14 as high-risk. Median follow-up was 18 months (range 1-116). At the time of the study 26 patients died and 43 experienced disease progression. Nine patients (7,6%) presented an abnormal FISH pattern. Four (two RA, one RCMD and one RAEB-1) showed a 7q22 deletion, one RA a 7q34 deletion, one RARS a 7q35 deletion, one RA the simultaneous deletion of bands 7q22 and 7q31, and two (one RCMD and one RAEB-1) the loss of all probes. In these last patients a commercial centromeric probe revealed 23% and 30% cells with one signal only, thus suggesting a cryptic -7. Repeated I-FISH analyses were carried out in thirteen patients: eight with a normal karyotype, two with a 7q22 deletion, one with a 7q34 deletion and two with -7. All experienced disease evolution. Thus, this last event occurred in 8/104 FISH normal patients and in 5/9 FISH abnormal patients. Interestingly, the RA patient with a 7q34 deletion developed a monosomy 7 fourteen months from clinical diagnosis and progressed to RAEB-1. In conclusion, our data suggest that in chromosomally normal patients FISH i) is a good tool to reveal cryptic 7q lesions; ii) provides an invaluable help for identifying patients at high risk of disease evolution; iii) reveals that a 7q deletion may be the first step in -7 development.

CO-042

IS PI-PLC 1 EXPRESSION A RELIABLE PREDICTOR OF RESPONSE IN LOW RISK MDS PATIENTS TREATED WITH LOW-DOSE AZACITIDINE? RESULTS OF A PROSPECTIVE MULTICENTRIC STUDY

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Background. The use of hypomethylating agents significantly modified the therapeutic approach to MDS patients, primarily in high-risk MDS patients. In low-risk MDSs the use of AZA hypomethylating agent is less understood. Epigenetic regulation of Phosphoinositide-Phospholipase C (PI-PLC) beta1 promoter and key molecules involved in the nuclear inositide signalling pathways seems to play an important role for the activity of AZA demethylating therapy. *Aims.* We prospectively evaluated the efficacy and safety of AZA low-dose in Low or Int-1 risk MDS patients who were symptomatic and/or unresponsive to previous treatments. In the same study we evaluated the molecular effects of AZA on PI-PLCbeta1 promoter methylation, in order to investigate a possibly correlation with the response to the drug. *Methods.* AZA was administered at a dose of 75 mg/mq/daily s.c for 5 consecutive days every 28 days for a total of 8 cycles. Final response was checked at the end of the 8th course. PI-PLC beta1 gene expression was evaluated on peripheral blood samples from patients at baseline, and monthly until the 8th cycle of AZA administration. *Results.* Between September 2008 and February 2010, 32 MDS patients with IPSS risk Low- or Int-1 were enrolled into the study. Most patients had a normal karyotype (63%) by metaphase cytogenetics, were BRC transfusion-dependent (81%), receiving a median of 4 units/mo, and were previously unresponsive to treatment including ESAs (69%). Twenty-six patients (81%) completed the treatment plan (8 cycles). The Overall Response Rate after the 8th cycle was 58% (15/26 pts) whereas 42% of patients maintained a stable disease; no patient progressed towards a high-risk MDS or AML. Five (19%) patients reached a complete remission whereas 10 (38%) achieved an hematological improvement. Transfusion independent was achieved in 8/26 patients (31%). The median duration of the response was 10 months; five patients maintain their response, that is CR in 2 cases (+24 and +30 months) and HI-E in 3 cases (+14, +25,+26 months) without any treatment or supportive therapy. No clinical or hematologic factor showed a correlation with the response. PI-PLCbeta1 gene expression was quantified in MDS patients at baseline and during AZA treatment. Results were calculated as a percentage ratio of PI-PLCbeta1 expression during AZA treatment compared to baseline. All but one patients (14/15) who achieved an hematologic response during treatment with AZA showed a statistically significant increase in PI-PLCbeta1 mRNA expression (P<0,001). In all patients the increase of PI-PLCbeta1 levels anticipated the clinical response obtained at the 8th cycle. *Conclusion.* The current results of our study showed that Aza low-dose schedule may be a safe and effective treatment for low risk MDS pts and may induce durable responses in some cases. The positive and significant correlation between the hematologic response and the PI-PLC 1 expression indicate that PI-PLC 1 could be a realiable marker of response to AZA in Low-Risk MDS patients.

CO-043

GENE EXPRESSION OF INOSITIDE-DEPENDENT SIGNAL TRANSDUCTION PATHWAYS IN MDS PATIENTS WITH DEL(5Q) TREATED WITH LENALIDOMIDE

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Introduction. Inositide signalling pathways are involved in cell growth, differentiation and apoptosis and play a role in the progression of MDS towards AML. In particular, an altered expression of nuclear PI-PLCbeta1 and activated Akt can lead to a deregulation of cell cycle processes, therefore affecting the survival of primary MDS cells. Moreover, we postulated an inverse correlation between PI-PLCbeta1 expression and Akt activation in MDS. Indeed, these processes are critical especially in low-risk MDS, that usually show a marked apoptosis and a low proliferation rate, which can be rapidly reversed, thus leading to a worse clinical status. Lenalidomide is currently used in the treatment of del(5q) low-risk MDS patients, to compensate and counteract their ineffective erythropoiesis. In fact, this drug has anti-angiogenic activity, suppresses inflammatory cytokine release, induces the erythroid differentiation and enhances the EPO receptor signalling. The exact molecular mechanisms underlying the effect of Lenalidomide in MDS cells are still unclear, even though it can target signalling pathways playing a role in the maintenance of the balance between apoptosis, proliferation and differentiation, such as PI3K/Akt. *Methods*. We studied 6 patients diagnosed with del(5q) Low-Risk MDS (IPSS: Low or Int-1) who were giv-

en Lenalidomide. We quantified the expression of several genes implicated in inositide signalling, such as PI-PLCbeta1 splicing variants and PI-PLCgamma1, as well as Cyclin D3 and Beta-Globin, in order to assess the effect of Lenalidomide on erythropoiesis and cell cycle. *Results*. In our case series, 4 out of 6 del(5q) Low-Risk MDS patients responded to Lenalidomide and showed an activation of erythropoiesis, in that Beta-Globin levels increased. Moreover, these subjects also displayed an activation of PI-PLCgamma1, which is associated with PI3K/Akt activation. As for the other 2 cases, patients early discontinued Lenalidomide for adverse events, and for these patients a clinical assessment of Lenalidomide effect was not possible. *Conclusions*. Our data support the hypothesis of a role for PI-PLCgamma1 activation during Lenalidomide treatment, and confirm the activation of erythropoiesis in responder patients. In fact, Lenalidomide increased the expression of genes specifically associated with erythropoiesis, like Globin genes, in our responder patients. Our results also indicate that both PI-PLCbeta1 splicing variants, as well as Cyclin D3, are not significantly affected by Lenalidomide, whereas PI-PLCgamma1 is specifically induced. Taken together, these results point to a specific activation of this pathway during the therapy and possibly pave the way to a larger investigation aiming to assess the role of these pathways in Lenalidomide response.

CO-044

ROLE OF INOSITIDE-DEPENDENT SIGNAL TRANSDUCTION PATHWAYS ON EPO-INDUCED EYTHROID DIFFERENTIATION IN LOW-RISK MDS PATIENTS

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Introduction. An impaired regulation of the PI3K/Akt axis is often associated with hematologic malignancies. Our group previously demonstrated PI-PLCbeta1 and Akt are inversely correlated in high-risk MDS. EPO treatment is currently used in the therapy of low-risk MDS, to compensate and counteract their ineffective erythropoiesis, although some patients do not respond to this treatment, or lose response. The activation of the EPO receptor has been linked to the activation of the PI3K/Akt/PI-PLCgamma1 axis, so that EPO could affect cell proliferation and apoptosis. *Methods*. Here we studied 16 MDS patients (IPSS risk: low or intermediate-1) and quantified the expression of several genes implicated in inositide signalling, that is PI-PLCbeta1 splicing variants and its downstream target Cyclin D3, as well as PI-PLCgamma1 and Beta-Globin, in order to assess the effect of EPO therapy. Moreover, we also studied the effect of EPO on Akt activation. Finally, to further investigate the role of PI-PLCbeta1 in erythroid differentiation, we exposed normal human CD34+ cells to EPO and analyzed the effect of PI-PLCbeta1 overexpression on Beta-Globin and PI-PLCgamma1 expression, as well as on Akt phosphorylation. *Results*. 8 out of 16 patients (50%) showed a favourable response to EPO, which was associated with a specific induction of Beta-Globin gene expression within the first two months of therapy. As for PI-PLCbeta1 and Cyclin D3 gene expression levels, the PI-PLCbeta1/Cyclin D3 axis is down-regulated in EPO responder patients after 3-4 months of therapy. Moreover, not only Akt phosphorylation, but also PI-PLCgamma1 gene and protein expression increased during EPO treatment, therefore confirming the activation of the Akt/PI-PLCgamma1 pathway in EPO responder patients. Finally, in normal CD34+ cells induced to erythroid differentiation, PI-PLCbeta1 overexpression abrogated both EPO-induced Akt phosphorylation and Beta-Globin expression. *Conclusions*. In our EPO responder patients the PI-PLCbeta1/Cyclin D3 axis is down-regulated after 3-4 months of therapy. This is consistent with previous findings showing that PI-PLCbeta1, after an early transient increase, is down-regulated in primary human erythroblasts treated with EPO for up to 96 hours, therefore suggesting that PI-PLCbeta1 could be required at the beginning of erythroid differentiation but is dispensable, if not inhibitory, at later stages. These results, along with the fact that EPO responders displayed a specific phosphorylation of Akt, suggest that PI-PLCbeta1 can act as a negative regulator of erythroid differentiation and confirm the involvement of the Akt/PI-PLCgamma1 pathway in EPO signalling, therefore contributing to the comprehension of the effect of EPO in low-risk MDS and possibly paving the way to the identification of MDS patients at higher risk of refractoriness to EPO treatment.

CO-045

A STANDARDIZED MORPHOLOGICAL PANEL IMPROVES ON THE EVALUATION OF DYSPLASTIC FEATURES IN MYELODYSPLASTIC SYNDROMES

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Introduction. The revised 2008 WHO classification confirmed minimal morphological criteria of myelodysplastic syndrome (MDS) diagnosis: at least 10% of bone marrow cells of at least one hematopoietic cell lineage must show unequivocal dysplasia to be considered as dysplastic. However, a structured and reproducible approach for the precise recognition of BM dysplasia is still missing, and the relationship between cytopenia and dysplasia needs to be clarified. The aims of this study were to identify a panel of reproducible morphological criteria associated with MDS useful for a correct application of WHO classification and to evaluate the prognostic relevance of the single morphological abnormalities and of the total lineage dysplasia as well as of the degree of dysplasia. *Methods*. We retrospectively examined the cytological features of BM smears from 429 MDS patients previously classified according to FAB criteria, 214 patients with hyporegenerative anemia and 74 healthy subjects. By counting 100 cells for the erythroid and granulocytic lineages and at least 20 megakaryocytes and classifying them for their dysplastic changes, a panel of dysplastic features showing a better sensitivity and specifity for MDS identification was developed. The morphological panel including 26 dysplastic features (12 erythroid, 8 granulocytic and 6 megakaryocytic) was employed to reclassify MDS patients by 2008 revised WHO proposal using the 10% threshold to record dysplasia in the erythroid and granulocytic lineages and the 25% threshold for dysmegakaryopoiesis. *Results*. Three hundred and one MDS cases were correctly reclassified, with a between-investigators and within-investigator agreement of 92% and 95% respectively. In MDS without an increase of BM blasts, Kaplan Meier estimates of overall survival (OS) and leukemia-free survival (LFS) showed that all patients with multilineage dysplasia had a significantly worse outcome, independently of the number of cytopenias (P=0.03 and P=0.0005 respectively). Some morphological abnormalities, i.e. erythroblast irregular nuclear edges or multinuclearity, granulocyte hypo-agranularity, small binucleated megakaryocytes, were associated with poor outcome, and total granulocytic or megakaryocytic dysplasia showed a significant independent unfavorable prognostic value (P=0.0004 and P<0.0001 respectively). Also the degree of granulocytic or megakaryocytic dysplasia, estimated based on percentage of dysplastic cells, was found to have a significant effect on OS (P=0.002 and P=0.0003 respectively). *Conclusions*. The definition of BM dysplasia with a standardized morphological panel that improves the objectivity and reproducibility of microscopic analysis is useful for a correct application of WHO classification as well as for the differential diagnosis between MDS and other cytopenias. Prognostic systems including the evaluation of the degree of BM dysplasia should be adopted for clinical decision-making.

CO-046

SELECTION BY SEVERE HYPOXIA OF REPOPULATING PROGENITOR CELLS IN PRIMARY MDS BONE MARROW CELL CULTURE

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Introduction. Myelodysplastic Syndromes (MDS) are clonal disorders. However, whether the transforming event occurs in a myeloid committed cell or in earlier (stem) progenitor it is still not ascertained. Evidence has been accumulated in both senses, but we suppose that MDS initiating cells must be capable of sufficient repopulating capacity to perpetuate the disease. In this study, we evaluated the repopulating ability of hypoxia selected cells in primary MDS bone marrow cultures and characterized the *stemness* of MDS maintaining cells. *Methods*. We evaluated 26 MDS cases of different WHO subtypes (9 RCMD,7 RAEB,5 AL/post MDS, 5 RA). Mononuclear bone marrow cells were isolated after gradient centrifugation and cultured in RPMI 1640 medium supplemented

with 20% FBS and a cocktail of cytokines (TPO, FLT3-L,SCF,IL-3). Cells were incubated and selected in Ruskin Concept 400 anaerobic incubator, in severe hypoxia conditions by flushing with a performed gas mixture (0,3 % O2,5%CO2,95% N2), for 10-13 days (LC1) and daily counted (Trypan blue). The stem and progenitor cell potential of these cultures at different times of incubation was explored by transferring cells to growth-permissive secondary cultures in normoxia (LC2),with SCF,G-CSF,IL-6,IL-3, according to the Culture-Repopulating Ability assay methodology (Leukemia, 14:735-9, 2000). At the same time, we evaluated clonal potential of selected cells by semisolid methylcellulose colony assay. The phenotype of hypoxia selected cells was evaluated by determination of the surface expression of CD34,CD38,CD117,CD133. When possible, we analyzed by FISH the presence of chromosomal aberrations in MDS cells before and after hypoxia selection, in order to see whether hypoxia- selected repopulating cells belonged to the neoplastic clone. *Results*. The hypoxic culture system allowed selection of a minute cell population: in 26/26 cases viable cell number was decreased of one log after 10-13 days. Only 9/26 cases showed a significant repopulating ability at day 17 of LC2. In the other 17/17 cases, repopulating ability was apparently absent. Parallel formation of CFU in semisolid cultures was observed In twelve cases analyzed, we observed after hypoxia selection a reduction of CD34+ cells; and within this population, in 8/12 cases we could select a population of CD34+ /CD38- cells, that confirmed the selection of primitive progenitor cells. In one case (characterized by 8 trisomy), we confirmed by FISH the presence of this chromosomal aberration in hypoxia-selected cells, although in this case no repopulating ability in LC2 was present. *Conclusions.* We demonstrate that it is possible to select by severe hypoxic conditions primary MDS progenitor cells with repopulating ability. More advanced MDS cases with higher percentage of blasts in the bone marrow did not show significant numbers of repopulating cells after selective hypoxic conditions.

CO-047

ANGIOGENESIS, APOPTOSIS, PROLIFERATION: IMMUNOHISTOCHEMICAL ANALYSIS IN MYELODYSPLASTIC SINDROMES AND IDIOPATIC DYSPLASIA.

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Introduction. Myelodisplastic syndromes (MDS) are clonal disorders of haematopoietic stem cells characterized by a maturation defect in progenitor cells, peripheral cytopenia and clonal instability with enhanced risk to transform into acute myeloid leukaemia(AML). When WHO criteria for the diagnosis of MDS are not met, there are two other conditions to consider: idiopathic cytopenia of undetermined significance (ICUS) and idiopathic dysplasia of uncertain significance (IDUS). The term IDUS was proposed to identify those cases where dysplasia is observed in the bone marrow (>10% in one or more lineages). Since the "IDUS-state" can be stable for years without developing an MDS or AML, it is particularly important to distinguish these categories. The aim of this study was to identify by immunohistochemistry biological markers able to differentiate these two subgroups. *Methods*. Bone marrow (BM) paraffin embedded biopsies from 8 patients with MDS (RA, RCMD, RAEB1, RAEB2) and 6 with IDUS were compared with 4 controls. The main clinical features of the patients were: 64% male with a median age of 68 years (range 57-82 years). The mean value of haemoglobin, white blood cells (WBC) and platelets was respectively 10.5 gr/dL(±0.6), $3.5x10^6$ (±0.6), $86x10^3$ (±24.8). The following antibodies were used to assess angiogenesis: VEGF, KDR/flk-1, MMP-9, CD34/QBEND1. CD34 antibody was useful also to determine BM vascularization. Ki-67, Bcl-2 and p53 antibodies were tested to evaluate proliferation and apoptosis, while erythroid precursors were detected by CD71. Antibody expression was evaluated as percentage and absolute number of positive cells in a total of eight consecutive areas at 400x magnification. To avoid any bias related to variations in BM cellularity, we normalized the expression calculating the "antibody expression index" (% BM cellularity x % Ab positive cells/104). *Results.* Expression of both KDR and p53 was similar comparing the three different groups (MDS, IDUS, controls), while the mean expression of CD34 and VEGF was higher in MDS. Bcl2, Ki67, CD71 and MMP9 appeared similarly expressed in MDS and controls, while in IDUS group their expression was lower. Statistical analysis showed a significant statistical difference for MMP9 (p 0.014), CD34 (p 0.05) and CD71(p 0.06), but after normalization for cellularity statistical significance was maintained only for MMP9 (p 0.01) and CD71 (p 0.01). CD34 and MMP9 expression resulted statistically significant also comparing MDS group vs ICUS and control groups. Vascularization was increased in 50% of patients with MDS, while it was normal in the other groups. *Conclusion.* Morphological approach is essential to distinguish MDS from IDUS; evaluation of biological markers by immunohistochemistry can be helpful to perform a differential diagnosis between MDS and ICUS, but further studies are warranted.

CO-048

MECHANISMS OF CELLULAR RESISTANCE TO AZACITIDINE IN MYELODYPLASTIC SYNDROMES

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Backgorund. Azacitidine is a pyrimidine nucleoside analogue of cytidine, a nucleoside present in DNA and RNA and is the standard treatment for myelodysplastic syndromes (MDS). A large number of studies performed to evaluate response to chemotherapeutics like cytosine arabinoside or gemcitabine, have demonstrated the importance of the expression levels of the enzymes involved in the metabolism of nucleoside analogs and their correlation with response to treatment. Despite the interest in azacitidine, little is known about its intracellular metabolism and scanty data are available. Clinical responses to azacitidine are heterogeneous and there is a need to identify molecular markers that could predict and/or monitor the efficacy of this therapy. A better understanding of the biological mechanisms involved in the activation and DNA uptake of azacitidine is therefore necessary to possibly predict responses in MDS patients. *Objective.* To measure the expression levels of the 5 enzymes involved in the azacitidine metabolism and to correlate gene expression with the response to azacitidine. Thirty-three MDS patients treated with azacitidine were included in the study. Of them, 12 patients were responders and 21 non-responders. *Methods.* Gene expression was assessed with quantitative PCR, using an ABI GeneAmp® 5700. Methylation of UCK1 was evaluated by methylation specific PCR using specific primers. The UCK1 coding region was sequenced by Sanger method. *Results.* Gene expression of hENT1, UCK1, DCK, RNR1 and RNR2 was compared between responders and non-responders. Individually, none of the genes was differentially expressed between responders and non-responders. Nevertheless, a close to significance trend for lower expression in non-responders was shown for the UCK1 gene (P=0.076). In order to verify whether the presence of aberrant methylation could impact in UCK1 gene expression, we measured the methylation status of a putative CpG island in the gene promoter region. The CpG island was unmethylated in all the samples analyzed, including responders and non-responder cases, as well as healthy donors. Moreover, to verify whether the difference in gene expression could be due to nucleotide variants in the gene sequence, we sequenced the UCK1 coding region. Preliminary results have shown the presence of common SNPs, although their relation with treatment response has not been investigated yet. *Conclusions.* Our results show that absence of clinical response to azacitidine could be related to a low expression of UCK1. This is most likely caused to a lower activation of the azacitidine monophosphorylation by UCK1 in non-responders patients. The genetic variation present in the coding region of UCK1 could offer new clues to explain the observed results. The methylation status of the UCK1 gene promoter could not affect gene expression. Further studies on larger series of cases are needed to confirm these results which could help driving therapeutic decisions.

Chronic Myeloid Leukemia

CO-049

DELAYED CYTOGENETIC RESPONSES AND REDUCED RATE OF MAJOR MOLECULAR RESPONSES ASSOCIATED TO INCREASED BODY MASS INDEX AT BASELINE IN CHRONIC PHASE CHRONIC MYELOID LEUKEMIA PATIENTS TREATED WITH IMATINIB

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Introduction. Obesity, measured as body mass index (BMI), has been identified as a possible risk factor for the onset of several solid tumors as well as for chronic myeloid leukemia (CML). To date, no correlations have been reported in this latter disease between BMI at baseline and response to targeted therapies. We refer here on the impact of BMI on clinical response in CML. *Methods.* 339 chronic phase (CP) CML patients treated with imatinib entered the study: 142 patients first received interferon alpha outside clinical trials and then were switched to imatinib for failure. For this group of patients, BMI was collected at the time of start of imatinib. The remaining patients were consecutively treated with imatinib first-line from January 2000 onward. BMI was defined as the individual's body weight divided by the square of his of her height and patients were categorized according to WHO into four categories: underweight (BMI < 18.5), normal weight (BMI 18.5-< 25), overweight (BMI 25-<30) and obese (BMI ≥ 30). All patients were followed according to ELN guidelines. We also analyzed 25 CP-CML patients treated frontline with nilotinib. *Results.* 156 patients (46%) were categorized as underweight/normalweight, while 183 patients (54%) were classified as overweight/obese. BMI increased with age, with a median age of 29 years in underweight category, 43.4 years in normal weight, 54.9 years in overweight and 62.4 years in obese patients (P=0.001). We did not reveal statistically significant association between BMI and prognostic risk stratification at baseline, even when we used new EUTOS score, or type of transcript. No statistically significant difference was revealed in terms of overall CCyR rate: 87% for underweight/normal weight categories compared to 84% for overweight/obese group (P=0.34). If compared to patients with low BMI (< 18.5-25), patients with increased BMI (> 25-40) at diagnosis who received imatinib, showed a significantly longer median time to reach CCyR (6.8 months vs 3.3 months, P=0.01), a reduced rate of MMR (77% vs 58%, P=0.01) which was also achieved in a longer median time (29 months compared to 14 months, P=0.03). At 18 months, molecular kinetics revealed that median BCR-ABL/ABL ratio was 0.6% IS (range 0.001%-2%) in underweight/normal weight group compared to 1.6% IS (range 0.01%-3%) in overweight/obese category (P=0.01). Conversely, no differences were revealed with respect to BMI in patients treated frontline with nilotinib and also patients with increased BMI obtained rapidly CCyR and MMR, with an incidence similar to that of underweight/normal weight patients. *Conclusions.* These results suggest that CML patients with increased weight at baseline should be followed and carefully monitored if treated with standard dose imatinib frontline for a possible early switch or, as an alternative, should preferably be candidate to receive second generation TKI as a first line therapy.

CO-050

HIGH EUTOS SCORE IS PREDICTIVE FOR ADVERSE OUTCOME IN EARLY CHRONIC PHASE CML PATIENTS TREATED WITH NILOTINIB-BASED REGIMENS

Castagnetti F,[1] Gugliotta G,[1] Palandri F,[1] Breccia M,[2] Levato L,[3] Stagno F,[4] Rege Cambrin G,[5] Luciano L,[6] Specchia G,[7] Abruzzese E,[8] Gozzini A,[9] Martino B,[10] Capucci A,[11] Intermesoli T,[12] Tiribelli M,[13] Cedrone M,[14] Cavazzini F,[15] Soverini S,[1] Testoni N,[1] Saglio G,[5] Martinelli G,[1] Pane F,[6] Alimena G,[2] Rosti G,[1] Baccarani M[1]

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Introduction. The outcome of Ph+ chronic myeloid leukemia (CML) has been significantly improved by the introduction of tyrosine kinase inhibitors (TKIs). Nilotinib (NIL) is a 2° generation TKI with superior efficacy to imatinib (IM), approved as frontline therapy of CML. Until recently, the prognosis of CML patients has been evaluated using prognostic scores developed in the chemotherapy and interferon era. The EUTOS score, a new scoring system based on the analysis of a large cohort of IM-treated CML patients in early chronic phase (ECP), has been proposed by the European Leukemia-Net (ELN); the variables included are basophils percentage and spleen size. *Methods*. The aim of the present analysis was to investigate the prognostic value of the EUTOS score in a cohort of ECP CML patients treated frontline with NIL-based regimens. The patients were enrolled in two phase 2 studies conducted by the GIMEMA CML WP (ClinicalTrials.gov. NCT00481052 and NCT00769327) or were treated with NIL as initial treatment in Bologna. Definitions: complete cytogenetic response (CCgR) was defined as the absence of Ph+ metaphases over at least 20 metaphases examined by conventional banding analysis or <1% BCR-ABL+ nuclei over 200 nuclei examined by I-FISH; major molecular response (MMR) was defined as BCR-ABL <0,1%IS; failure was defined according to 2009 ELN criteria; progression was defined as the transformation to accelerated or blastic phase; deaths, for any reason and at any time. All the calculations were performed according to the intention-to-treat principle. *Results*. 215 patients were included; median age 53 years (range 18-86). The patient distribution according the different scoring systems was as follows: 95% low and 5% high EUTOS score; 38% low, 44% intermediate and 18% high Sokal score; 39% low, 56% intermediate and 5% high Euro score. The median follow-up was 29 months (range: 18-43 months). The cumulative CCgR rate was 93%; the cumulative MMR rate was 89%; the failure-free survival was 90%; the progression-free survival 93% and the overall survival was 94%. No difference in the cumulative CCgR rate at any time was observed according to EUTOS score, but patients with low EUTOS score achieved a significantly higher cumulative rate of MMR (91% versus 60%, P=0.01). Interestingly, the patients with low EUTOS score had higher failure-free survival (91% versus 70%, P=0.02), higher progression-free survival (94% versus 80%, P=0.05) and higher overall survival (95% versus 79%, P=0.04). The Sokal score was able to predict differences in terms of MMR at any time and failure-free survival, but not in terms of progression-free survival or overall survival. The Euro score failed to detect any response and outcome difference. *Conclusions*. In a cohort of CML patients treated with NIL-based regimens as frontline therapy, the prognostic predictive ability of EUTOS score resulted superior to Sokal and Euro score. *Acknowledgments*. ELN, COFIN, Bologna University, BolognAIL.

CO-051

THE AXIS SHP-1/SHP-2 MODULATES VEGFR2 SIGNALING IN CML CELL LINES: VEGFR2 DEREGULATION MAY BE IMPLICATED IN IMATINIB (IMA) RESISTANCE

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Introduction. IMA targeted inhibition of BCR-ABL is considered the standard front-line therapy of CML. However, although the vast majority of patients respond to the therapy, primary or acquired resistance to IMA may occur during treatment. We previously show that the tumor suppressor tyrosine phosphatase SHP-1 plays a key role in BCR-ABL-independent IMA resistance modulating the activation signals that SHP-2 receives from both Bcr/Abl and membrane receptor tyrosine kinases in both CML cell line and patients.[1] Several groups show that the signaling from VEGFR-2 is necessary for proliferation, chemotaxis and cell survival of several tumor systems. The elucidation of the BCR-ABL independent regulatory pathway in IMA resistant CML cells is important to develop new therapeutic strategy in the case of IMA resistance. *Methods*. In this study we investigated the role of SHP-1 and SHP-2 in VEGFR-2 signaling in KCL22 Ph+ cell lines (IMA sensitive/KCL22-S and IMA resistant/KCL22-R). To evaluate if VEGFR-2 interact with SHP-1/SHP-2, we perform specific co-immunoprecipitation assay. Moreover, we carried out WB experiments to evaluate the phosphorylation status of VEGFR-2 protein in our CML system. To further characterize the functional role of VEGFR-2 activation in CML cell lines, we cultured KCL22 in the presence of 2 µmol/L of VEGFR-2 inhibitor sorafenib. *Results*. WB analysis showed that VEGFR-2 is equally expressed in KCL22S and KCL22R cell lines. In addition, we demonstrated that VEGFR-2 forms a complex with SHP-1 and SHP-2 in sensitive KCL22S cell line. In particular, VEGFR-2 interacts only with SHP-2 in KCL22R cell line, in which SHP-1 is down-regulated. Ectopic SHP-1 expression was able to establish VEGFR-2/SHP-1 interaction also in resistant cell line. Recently, Bhattacharya demonstrated that SHP-1 regulates negatively VEGFR-2 signaling by dephosphorylation of specific tyrosine residues. Thus, we hypothesized that SHP-1 might modulate negatively the angiogenic signals in KCL22S cell line, through dephosphorylation of VEGFR-2. Concomitantly, VEGFR-2 signaling through SHP-2 may be significantly activated in resistant cell line and CML patients lacking of SHP-1 expression. Indeed, we demonstrated that VEGFR-2 activation sites (Y996 and Y1059) remain phosphorylated after IMA treatment only in KCL22-R but not in KCL22-S cell line and that the ectopic SHP1 expression in resistant cell line is associated with dephosphorylation of the same sites. To better clarify the functional role of VEGFR2 activation in IMA resistance, we treated KCL22S and R with the VEGFR-2 inhibitor sorafenib. Sorafenib treatment induced apoptosis in 60% of KCL22R, whereas it does not produced effects on KCL22S viability. We did not detect any synergistic effects of sorafenib and IMA in resistance cell line. *Conclusions.* Our data indicate the involvement of VEGFR-2 in the pathways of Bcr-Abl independent IMA resistance and that the axis SHP-1/SHP-2 may modulate VEGFR2 signaling in CML cell lines.

Reference

1. Esposito N, et al. Blood 2011.

CO-052

FRONTLINE NILOTINIB 400 MG BID IN EARLY CHRONIC PHASE CHRONIC MYELOID LEUKEMIA: STABLE RESULTS BEYOND 4 YEARS - THE GIMEMA CML WP TRIAL CML0307

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Background. Nilotinib (NIL) is a potent and selective BCR-ABL inhibitor approved for the first line treatment of CML. The ENESTnd trial (24 months follow-up), showed higher and faster molecular responses and lower rates of progressions to accelerated-blastic phase of NIL vs. imatinib. With imatinib 400mg (IRIS trial), the rate of events and progression to AB/BP were higher during the first 3-4 years. Consequently, a confirmation of the durability of responses to NIL after 4 years is extremely relevant. *Methods*. The aim of the present analysis was to evaluate the long-term outcome of patients treated with NIL 400mg BID as frontline therapy. A multicentre phase 2 trial was conducted by the GIMEMA CML WP (ClinicalTrials.gov. NCT00481052). Four years minimum follow-up data for all patients will be presented. Definitions: MR3.0 (Major Molecular Response) as a BCR-ABL/ABL ratio <0,1%IS; MR4.0, undetectable transcript levels with ≥10,000 ABL transcripts; failures: according to the revised ELN recommendations; events: failures and treatment discontinuation for any reason. All the analysis has been made according to the intention-to-treat principle. *Results.* 73 patients enrolled: median age 51 years; 45% low, 41% intermediate and 14% high Sokal risk. The cumulative incidence of CCgR at 12 months was 100%. CCgR at each milestone: 96% and 92% at 12 and 24 months, respectively. The overall estimated probability of MR3.0 was 99%, while the rates of MR3.0 at 12 and 24 months were 85% and 82%, respectively. Two out of 73 patients never achieved a MR3.0, 1 who progressed to AB/BP (see below) and 1 in stable and confirmed CCgR at 36 months. Only 3 pts had a confirmed loss of MMR due to low adherence (all 3 still on NIL, 1 patient re-obtained a MMR). The overall estimated probability of MR4.0 was 79%, while the rates of MR4.0 at 12, 24 and 36 months were 12%, 27% and 30%, respectively. One third (22/73 pts) showed a stable MR4.0 (defined based on 3 consecutive MR4.0 samples 4 months apart). Only one patient progressed at 6 months to AP/BP and subsequently died (high Sokal risk, T315I mutation). The last daily dose was 600 mg or higher for 49 patients (67%). Six patients discontinued permanently NIL: 1 patient progressed to AB/BP; 3 patients had recurrent episodes of amylase and/or lipase increase (no pancreatitis); 1 patient had atrial fibrillation (unrelated to study drug) and 1 patient died after 32 months of mental deterioration and starvation (unrelated to study drug). During the fourth year of therapy (median follow-up of 45 months) no new events occurred; the estimated probability of overall survival, progression-free survival and failure-free survival was 97%, the estimated probability of event-free survival was 91%. *Conclusions.* Given the very low overall rate of failures and the stable molecular responses during the fourth year of NIL the outcome remains optimal for most of patients. *Acknowledgments*. European LeukemiaNet, COFIN, Bologna University, BolognAIL.

CO-053

CLINICAL RELEVANCE IN CHRONIC MYELOID LEUKEMIA OF DELETION AND INSERTION EVENTS IN THE TYROSINE KYNASE DOMAIN OF BCR-ABL

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Background. While the majority of CML patients achieve a response to the treatment with inhibition of TK, some of them show only suboptimal response (SO) or resistance to TKI treatment. One of the most frequent cause of resistance is the onset of point mutations at catalytic site of ABL. These mutations impair interactions of oncogenic BCR-ABL protein to Imatinib and, at least existent, also to second generation TKIs. We characterized the ABL deletions/insertions in 23 CML patients that showed Imatinib resistance. *Methods*. A total of 830 patients were treated with Ima and monitored by RTQ-PCR, according to ELN recommendations. In 225 SO or failure patients the ABL sequence of BCR-ABL was analyzed and deletions/insertions were confirmed by ARMS-PCR. Based on wild type BCR-ABL crystallography, mutated sequences were analyzed by homology modeling (HM) to generate 3D structures and to predict TKI bindings. *Results*. ABL mutations were detected in 118 out of 225 (52%) tested patients: 80% of them reported single aminoacid substitutions and the remaining patients showed ex7-9 ABL deletion (19%) or insertion (1%). No differences were observed in terms of age (median 52±18, range of 26-78), sex and WBC count between patients with point mutations and with deletions or insertions. HM analysis reveals that ABL mutations induced frameshift alterations in 14 out of 23 patients, causing early ABL proteins truncations with loss of 2-3/5 Ima H-bonds and 2/2 Dasa H-bonds. Among these 14 patients: 6 switched to Dasa, 3 of which died and the others switched to Nilo and are still alive in CCyR; 8 continued Ima and are still alive, but not two. Two patients with complex BCR-ABL rearrangements (ex4-9 ABL deletion and ex13 BCR insertion) do not produced ABL fusion proteins and lost the mutated clone during Ima treatment. Moreover, we observed ABL D363-R386del in 4 patients, in which HM reveals the loss of A-loop and one Ima H-bond, but no Dasa H-bonds. Three out of 4 patients continued Ima treatment and are still alive. The last one switched to Dasa and acquired a new ABL frameshift mutated clone losing all Dasa H-bonds. Thus, patient started to be treated with Nilo without MMR achieving and dying after TMO. None change in TKI interactions was observed in the case of ABL t1143del causing a C-terminal truncated protein and in the case of a simultaneous ABL P293-1 insertion and Lys294Gln exchange. In this last case, the ins293-1P clone became predominant during Ima therapy, whereas it disappears during Dasa treatment and now the patient is still alive after TMO. *Conclusions*. The TK domain is significantly altered in the majority of the detected ABL deletion/insertion events and Ph+ mutated cells are prone to add further alterations giving survival advantages to the leukemic clone. In conclusion, screening for deletion/insertion ABL mutations associated with homology modeling analysis to predict TKI binding, may be an experimental tool to help tailoring therapy for patients with CML.

CO-054

FUNCTIONAL ANALYSIS OF BONE MARROW (BM) NICHES FOR THE REGULATION OF TYROSIN KINASE INHIBITOR (TKI) ACTIVITY ON PHILADELPHIA POSITIVE (PH+) CELL LINES AND CD34+ PROGENITOR CELLS DERIVED FROM PATIENT WITH CML

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Introduction. The importance of tumor microenvironment for cancer progression is becoming widely recognized in recent years. The Bone Marrow (BM) is a dynamic microenvironment with high concentration of growth factors and cytokines necessary for haematopoiesis, making it a highly permissive zone for cancer haematopoietic staminal cell homing and survival. It is possible that the same factors that modulate hematopoiesis promote leukemogenesis, enhance blast survival and make them resistant to treatment within the BM microenvironment. In the era of molecular target therapy, whereas Imatinib has shown a cumulative best complete cytogenetic response rate of 82% and an estimated event free survival at 8 years of 85%, several *in vitro* data have confirmed that Ph+ CD34+ progenitor cells crammed in BM niches are resistant to TKI treatments. We attempted to define BM microenvironment markers that nurture and determine stem cell fate in leukemia associated-niches. *Methods.* We treated Ph+ K562 cell lines and primary CD34+ BM cells derived from untreated CML patients with a dose range of TKIs (0-100 μM) in the presence of a monolayer of human BM mesenchimal stromal cell line (HS-5) or HS5 conditioned media (HCM), to assess the role of BM niche in the regulation of TKI responsiveness. Selected experiments were conducted by adding to the *in vitro* culture several cytokines, known to be critical mediators of stromal/leukemic cell interaction. *Results*. We demonstrated that BM stroma enviroment significantly protects K562 cell line from TKI-induced apoptosis. Indeed, we demonstrated that IC50 value (calculated on either proliferation or apoptotic assay) of Imatinib, Nilotinib and Dasatinib increased of almost one log when leukemic cells are exposed to HS5 or HCM. We detected more than 60% of proliferating cells and less than 40% of apoptotic cells when K562 cell line has been observed after treatment with 300nM Imatinib, 30 nM Nilotinib or 3nM Dasatinib in the presence of HS5 or HCM. Moreover, we prove that a significant TKI-resistance could be achieved also by Ph+ CD34+ primary CML cells exposed to HS5 or HCM. Interestingly, we demonstrated that TKI treatment is not able to reduce STAT3p when K562 cell line is also exposed to HS5 cell line. Thus, we evaluate if the major stromal-derived cytokines, i.e. SDF-1, SCF, IL3, IL6, IL8, G-CSF and GM-CSF might be responsible for the regulation of TKI responsiveness in Ph+ cell line treated with TKIs. We demonstrated that the applied cytokines did not significantly modify TKI-induced apoptosis in either K562 or CD34+ primary CML cells. Thus, we are currently screening with a multiparametric approach all known soluble factors secreted by HS-5 cell line. *Conclusions*. Taken together, these findings indicate that BM-derived stroma cell line produces a strong effect on the regulation of TKI responsiveness in Ph+ CML cells by both a direct cell-to-cell contact and exposition to soluble factors.

CO-055

EXPERIMENTAL AND COMPUTATIONAL CHARACTERIZATION OF AMINO ACIDIC RESIDUES IN THE BCR-ABL KINASE DOMAIN CONTRIBUTING TO TKI RESISTANCE IN PATIENTS WITH CHRONIC MYELOID LEUKEMIA

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Introduction: Suppression of BCR-ABL1 catalytic activity by different tyrosine kinase inhibitors (TKIs) has dramatically improved the natural history of Chronic Myeloid Leukemia (CML). Despite the unparalleled results achieved by TKIs, 15 to 30% of CML patients (pts) become resistant to these drugs, in part because of point mutations in the BCR-ABL catalytic domain. These substitutions are not randomly distributed in the BCR-ABL kinase sequence as only one (T315) of five residues (E286, T315, M318, I360, D381) potentially involved in TKI interactions is the object of point mutations in pts failing these drugs. *Methods.* We generated eight FLAG-tagged-BCR-ABL constructs displaying conservative or non-conservative mutations in each of the four residues (E286, M318, I360, D381) that have never been found mutated in TKI-resistant pts. We also engineered BCR-ABL wild-type and T315I as a control. Every construct was lentivirally transduced in Ba/F3 cells and evaluated for expression, kinase activity and transforming potential. We subsequently analyzed the response of multiple BCR-ABL mutants to first (Imatinib; IM), second (Dasatinib; DAS) and third (Ponatinib; PON) generation inhibitors. We also performed 50ns Molecular Dynamics (MD) simulations of different BCR-ABL kinase domains (KD) in a simulated aqueous environment using the GROMACS package with AMBER force field. These simulations were repeated after binding to IM, DAS and PON, to analyze the average distance of hydrogen-bonds linking each inhibitor with its critical residues within the BCR-ABL KD. *Results.* Among all conservative and non-conservative mutations, I360T was the only substitution capable of maintaining BCR-ABL kinase activity and transforming potential. However, BCR-ABLI360T remained sensitive to all tested TKIs. MD simulations revealed unwinding of the C helix with covariance web analyses showing increased motility of the C-lobe in BCR-ABLT315I kinase domain. The I360T mutation induced an upward displacement of the C helix and perturbed the stability of the ATP-binding pocket. Increased average distances in hydrogen-bond interactions between BCR-ABLT315I and IM explained the drug's failure against this mutant. On the contrary, modest variations in hydrogen-bond interactions between IM, DAS or PON and both wild-type and I360T BCR-ABL were consistent with their sensitivity to the three TKIs. *Conclusions.* Our findings provide a mechanistic explanation for the efficacy of Ponatinib on the BCR-ABLT315I mutant. We also demonstrate that, within the ABL KD, E286, M318 and D381 are critical residues to preserve BCR-ABL kinase activity and oncogenic potential. Designing small molecules targeting structurally critical amino acidic residues within the catalytic domain of protein kinases may be of therapeutic significance for the personalized treatment of multiple solid and hematologic malignancies.

CO-056

HIGH SOKAL RISK IN CHRONIC MYELOID LEUKEMIA PATIENTS AT DIAGNOSIS SHOWED THE ELEVATED EXPRESSION OF FBP1, A KEY-ENZYME OF GLUCONEOGENESIS

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CML patients are historically stratified according to their Sokal risk score, which for more than 30 years has been regarded as the most significant prognostic factor in this hematological malignancies. The putative genetic and/or genomic basis driving this stratification are still not known. Here, we explored the molecular mechanisms associated to the CML patients stratification according to the Sokal risk score, by analyzing the gene expression profiling (GEP) of the CD34+ cell fractions obtained at diagnosis from a cohort of high and non-high Sokal risk CML patients. 67 patients with previously untreated CML in chronic phase (CP) and enrolled in GIMEMA CML protocols provided highly enriched CD34+ cell fractions from peripheral blood. GEP was performed in order to identify genes most most differentially expressed between high and non-high Sokal risk patients. By GEP, 82 probe-sets, corresponding to 78 genes resulted significantly differentially expressed between high and non-high Sokal risk patients in a supervised analysis of gene profiles. A gene enrichment analysis of this profile showed that genes involved in the Wnt, in the Notch signaling pathways and in the response to hypoxia and oxidative stress resulted significantly overexpressed in the comparison between high and non-high risk patients. We focused our attention on genes involved in the glycolysis and gluconeogenesis metabolic pathways (FBP1, SEPP1, GSTM3, GSTT1, PRDX2, MPO). We validated by Real-time the de-regulated expression of these genes in a different set of newly diagnosed CP-CML patients, thus confirming that they are differentially expressed between high and non-high risk patients, with trends similar to those observed by GEP. Particularly interesting resulted a significantly higher expression in high Sokal risk patients of FBP1 (fructose 1,6 bisphosphatase), a key-enzyme of gluconeogenesis, together with a significant over-expression of genes coding for enzyme involved in gluthatione biosynthesis (GSTM3, GSTT1, and PRDX2). These data suggest that CD34+ cells obtained from high Sokal risk patients might exhibit an unexpected moderation of the glycolytic flux, mainly due to the over-expression of FBP1, which might cause a re-direction of the pathway into the pentose phosphate shunt. A similar metabolic reprogramming has been already described in imatinib resistant bcr-abl positive cell lines and is supported also by the over-expression in high risk patients of G6PDH (glucose 6 phosphate dehydrogenase) and TK (transketolase), which are key enzyme of the pentose phosphate shunt. Overall, our data demonstrate that the expression at diagnosis of sugar metabolic enzymes might drive the evolutive Sokal risk of CML patients. Supported by: European LeukemiaNet, BolognAIL, AIRC, Fondazione Del Monte di Bologna e Ravenna, FIRB 2006, PRIN 2008, Ateneo RFO grants, Project of integrate program (PIO), Programma di Ricerca Regione, Università 2007, 2009.

Cytogenetics and Molecular Analysis

CO-057

C-MYB TRANSACTIVATES THE EXPRESSION OF THE ERYTHROID HSA-MIR16-2 GENE

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Introduction. miR16-2 is overexpressed in CD34+ cells of patients with Polycythemia Vera (PV) versus normal donors. We recently demonstrated that forced expression of miR16-2 in normal CD34+ cells stimulated erythroid maturation while its down-regulation impaired erythropoiesis both *in vitro*, in PV CD34+ cells, and in vivo. These data identified miR16-2 as a positive regulator of the erythropoiesis and linked the abnormal expansion toward the erythroid lineage to the overexpression of miR16-2 in PV patients. However, the mechanisms underlying miR16-2 overexpression are still unknown. This project aims to identify the transcription factors (TFs) regulating miR16-2 expression in normal and PV erythropoiesis. miR16-2 is an intronic miRNA. A functionally defined promoter of miR16-2 has not been characterized yet. Therefore, based on (1) the identification of a RNA pol II-enriched region overlapping SMC4 promoter for miR16-2 expression and (2) the correlation between the expression levels of miR16-2 and SMC4 host gene, we focused our attention on the TFs regulating SMC4 expression. *Methods.* SMC4 gene promoter was screened to identify putative binding sites for TFs known to be involved in erythroid differentiation, such as c-myb, KLF1 and GATA1. Next, we cloned the SMC4 promoter region between ~20 bp downstream and 1200 bp upstream the Transcription Start Site into the pXP1 plasmid, upstream to the promoterless luciferase reporter gene. HEK293 cells were transfected with the pXP1 vector carrying the Luciferase gene under the SMC4 promoter control and increasing amounts of plasmid coding for c-myb, GATA1 or KLF1. Luciferase activity was measured in duplicate and signals were normalized for transfection efficiency to the internal Renilla control. *Results.* Our data demonstrated that increasing levels of c-myb protein expression are able to transactivate SMC4 promoter-driven luciferase expression. In fact, increasing c-myb expression levels determined a dose-dependent increase in SMC4 promoter-driven luciferase activity (735+/-196, 995+/-286 and 1759+/-474 for 100, 200 and 400ng of c-myb-coding plasmid respectively, versus 590+/-190 for the empty plasmid control; average+/-SD values). Therefore, the c-myb-driven SMC4 promoter transactivation trend demonstrated the involvement of c-myb in SMC4/miR16-2 expression control. Further experiments will elucidate the role of GATA1 and KLF1 in this process. *Conclusions.* In conclusion, our data demonstrated that c-myb is able to transactivate SMC4 promoter, by shedding for the first time some light on the transcriptional control of miR16-2/SMC4 in normal and PV erythropoiesis. We recently demonstrated that c-myb supports erythropoiesis by transactivating KLF1 and LMO2 expression. Our data point out SMC4/miR16 transactivation as a new pathway through which c-myb affects erythroid differentiation. However, further studies need to be performed to clarify this mechanism and its relevance in normal and PV erythropoiesis.

CO-058

CRIZOTINIB-RESISTANT NPM-ALK MUTANTS CONFER DIFFERENTIAL SENSITIVITY TO UNRELATED ALK INHIBITORS

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Introduction. The oncogenic fusion protein NPM-ALK, originated from the t(2;5)(p23;q35) translocation, is recognized to be the leading cause of about 70-80% of cases of Anaplastic Large Cell Lymphoma (ALCL). Nowadays, new effective ALK inhibitors such as Crizotinib, or, more recently, the phase II clinical trial AP26113, are available and open the possibility to successfully treat ALCL and other ALK related diseases. Previous experience in the treatment of CML with Imatinib and other Tyrosine Kinase Inhibitors (TKIs) revealed that the majority of patients who undergo relapse do so because of point mutations in the oncogene kinase domain or gene amplification. The aim of our work was to investigate the possible mechanism of resistance against Crizotinib and, in particular, to individuate point mutation in ALK kinase domain. Moreover, we checked the sensitivity of our Crizotinib resistant clones to two different ALK inhibitors: AP26113 and the structurally unrelated NPV-TAE 684. *Methods.* We cultured two different ALCL ALK+ cell lines, KARPAS299 and SUP-M2 in the presence of increasing doses of Crizotinib. We excluded ALK overexpression as the leading resistance mechanism, so we clonally sequenced ALK Kinase domain in the resistant populations. We cloned and transfected the identified mutations in Ba/F3 cells to investigate their biological role. Molecular modeling was used to analyze the structural mechanism of resistance. *Results.* A single aminoacid substitution became predominant at higher Crizotinib concentrations in both cell cultures. L1196Q, located near the ATP binding site, was found in KARPAS299 cells, while I1171N, known as an activating mutation in neuroblastoma, was present in 100% of SUP-M2 clones. We found that L1196Q substitution confers resistance to Crizotinib but not to AP26113 and NPV-TAE 694, while cells carrying I1171N mutation are resistant to all inhibitors. *Conclusions.* Starting with two human ALCL ALK+ cell lines we found as a possible cause of Crizotinib resistance two mutations located in different regions of ALK kinase domain. Knowledge about the appearance of these mutation in patients treated with Crizotinib and the possibility to overcome them using new TKI can be useful tools in the management of Crizotinib-resistant ALCL cases.

CO-059

TRANSLOCATIONS INVOLVING NUP98 AND MLL GENES TARGET CD34+ HEMATOPOIETIC CELLS IN ACUTE LEUKAEMIA

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Introduction. NUP98 and MLL, two promiscuous genes, are involved with diverse partners (at least 28 for NUP98 and over 100 for MLL) in myeloid and lymphoid leukemias. To investigate hematopoietic lineages affected by NUP98 and MLL changes in AML and ALL the present study used FICTION with simultaneous immunophenotyping and interphase FISH. *Methods.* Twelve cases were investigated: 7 with a NUP98 rearrangement and 5 with MLL involvement. NUP98 partners were NSD1 (1 RAEB), DDX10 and LOC348801 (2 AML), RAP1GDS1 (1 AML, 2 T-ALL) and an unknown partner in 1 AML. MLL partners were AF4 (2 B-ALL), AF9 (1 AML, 1 B-ALL), and FBP17 (1 AML). Monoclonal antibodies for FICTION studies on bone marrow cells were: anti-CD34, anti-CD133, anti-CD33, anti-CD13, anti-CD14, anti-glycophorin A , anti-CD19, anti-CD20, anti-CD3, anti-CD7. To detect NUP98 and MLL rearrangements we applied clones RP11-348A20 and CTD-3234F16, encompassing the entire NUP98, and clones RP11-832A4+RP11-861M13+RP11-77OJ1 for MLL. Immunophenotype (in red) and hybridization signals (in green) were simultaneously identified and counted under an Olympus fluorescence microscope with filter sets for Cy-3 (red) and FITC (green). At least 15 cells (range 15-160) were analysed for each antibody. Cytospins with bone marrow cells from two healthy donors were used as normal controls for each antibody. The cut-offs for NUP98 and MLL split were established at the upper limit from normal controls. *Results.* In all NUP98-positive patients FICTION showed that hematopoietic CD34+/CD133+ cells carried the NUP98 fusion while cells positive for differentiation antigens were differently implicated. In 1 RAEB and 3 AML cases, the NUP98 fusion was detected in myeloid precursors (CD33+, CD13+, CD14+) and erythrocytes (Glycophorin A+). In the RAEB case and in 2 AML cases, it was found in B (CD19+, CD20+) and T (CD3+, CD7+) lymphocytes. In 2 T-ALL only CD3+/CD7+ cells bore the NUP98 fusion. In all MLL+ patient FICTION showed that hematopoietic CD34+/CD133+ cells carried the MLL+ fusion. Downstream, t(4;11) in 2 B-ALL patients showed MLL splitting in CD33+ (1 case) and in CD19+ cells (both). The other cases, 1 AML with MLL-FBP17, 1 B-ALL and 1 AML with MLL-AF9, always showed myelomonocytes were involved .CD19 was implicated only in the patient with B-ALL. *Conclusions.* This study provided evidence that a CD34+ totipotent stem cell is affected in both NUP98+ and MLL+ leukemias. Besides CD34+ cells, myelo-monocytes, erythrocytes and lymphocytes are involved in NUP98+ AML. On the other hand, in NUP98+ T-ALL only T-lymphocytes are affected, showing the myeloid differentiation programme is excluded. Conversely CD33+ CD13+ and

CD14+ cells are affected in AF4/MLL and AF9/MLL positive B-ALL. This finding is consistent with mixed lineage leukemias and interestingly indicates myeloid differentiation is partially conserved in B-ALL. *Acknowledgments.* FCRP: 2012.0108.021

CO-060

ROLE OF NFIB IN NORMAL HEMATOPOIESIS AND MYELOPROLIFERATIVENEOPLASMS

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Introduction. The three-canonical BCR-ABL negative myeloproliferative neoplasms (MPN), Polycythemia Vera (PV), Essential Thrombocytosis (ET) and Primary Myelofibrosis (PMF) are chronic diseases, which share the risk of disease evolution to an acute leukemia. In MPN, the most representative molecular lesion is a substitution of a valine for a phenylalanine (JAK2V617F) in the auto-inhibitory domain of JAK2, resulting in the constitutive expression of the gene.This point mutation has an incidence of about 96% in PV, 65% in PMF and 55% in ET. Microsatellite studies on chromosome 9 show the presence of a uniparental acquired disomy (UPD) of the short arm (9p), where JAK2 is located, as a common defect in MPN (Kralovics et al., ExpHematol. 2002). In a cohort study, all the samples with the 9pUPD were found positive for the JAK2V617F mutation (Klampfl et al, Blood 2011). The Nuclear Factor IB (NFIB) gene is present in the 9pUPD region, where a mutational hot spot takes place. NFIB belongs to the NFI family of CAAT box binding transcription factors, consisting of 4 separate genes (NFIA, -B, -C, -X). NFIA is a post-transcriptional target of myelopoiesis regulator miR-223, which plays a key role in directing the HSC/HPC maturation/differentiation into the erythroid or granulocytic lineages (Fazi et. al. Cell 2005; Starnes et al. Blood 2009). Genomic alterations of NFIA were detected in about 2% of MPN patients (Bernard, Leukemia 2009). The gene expression levels of NFIB are higher in CD34+HSC/HPC isolated from 5 JAK2V617F+PV patients than in normal controls (Berkofsky-Fessler, Clin Cancer Res 2010). However, the role of NFIB in normal and pathological hematopoiesis has not been yet investigated. *Methods.* DNA, mRNA, and proteins were isolated from human myeloid cell lines (K562, Hel and UKE-1) and buffy coat from peripheral blood (PB) and bone marrow (BM) cells isolated from MPN patients or healthy donors. Gene dosage and gene expression level were measured by qRT-PCR. Statistical analyses were used to calculate differences among and between groups by one way ANOVA and two-way t-test. *Results.* our preliminary data shows that: i) NFIB locus is amplified and its expression is increased in myeloid cell lines harboring the JAK2V617F; ii) NFIB is barely expressed in mononuclear cells isolated from healthy donors PB (n=23), while its expression increased in PB cells isolated from PV (n=18, P=0.034) and ET patients (n=27, P=0.005), independently from JAK2V617F status; iii) increased gene dosage of NFIB , paralleling JAK2 gene amplification is detected in MPN patients (n=27, P<0,001); iv) gene expression level of NFIB positively correlates with platelets count in ET patients (n=17, P=0.008). *Conclusions.* our preliminary data show the de-regulation of NFIB expression levels related to MPN, thus suggesting its role in MPN pathogenesis or disease evolution and usage as a marker for MPN diagnosis and/or prognosis.

CO-061

PAX5/ETV6 ACTIVATES MIR-675 AND ITS IMMATURE PRECURSOR H19 GENE, WHICH IS PHYSIOLOGICALLY REPRESSED BY PAX5

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Introduction. The PAX5 gene is altered in 32% of pediatric BCP-ALL patients, but the role of these lesions is still poorly understood. The most recurrent translocation t(9;12) encodes for the PAX5/ETV6 gene. We previously performed a gene expression profiling of murine wild type (wt) pre-BI cells, demonstrating that PAX5/ETV6 affects the normal PAX5 pathway, exerting an opposite dominant effect on the endogenous gene, causing the down-regulation of genes physiologically activated by wt PAX5 and activating genes normally repressed by PAX5, such as the H19 gene. Here, we aim to investigate the molecular consequences of the overexpression of H19, which is the immature precursor of mir-675. *Methods*. Wt pre-BI cells and PAX5-/- pro-B cells were transduced by MIGR-PAX5/ETV6 vector. After sorting for GFP, cells were suspended in Trizol and total RNA was extracted and processed on the Affymetrix 430A2.0 gene chip. Primers and probes for HPRT (reference gene) and H19 were selected according to the Universal Probe Library Software Probe Finder (Roche). Reverse transcription with a miRNA-specific primer has been done using the TaqMan MicroRNA Reverse Transcription Kit (Life Technologies) and followed by real-time PCR with single assays for mmu-miR-675-5p and -3p and RNU6B snRNA (reference miRNA). The comparative Ct method has been applied for analysis. *Results*. Among genes up-regulated by PAX5/ETV6 with FC≥1.5, we recognized 15 PAX5-target probes. Strikingly, 11/15 are known to be repressed by wt PAX5, including the H19 gene. Its over-expression has been validated by RT-qPCR. The H19 transcript is an immature precursor of micro-RNAs mir-675-5p and -3p. By specific RT-qPCR, we demonstrated that in wt pre-BI cells, mir-675-5p and 3p were detected at basal but appreciable levels and PAX5/ETV6 cells showed a significant up-regulation of mir-675-3p (FC=7.94; t test, P=0.0022) and mir-675-5p (FC=5.72; t test, P=0.0117) respectively. We additionally investigated the expression of the H19 gene in PAX5-/- cells. As expected, we detected higher expression levels of usually PAX5-repressed genes in PAX5-/- control cells vs wt control cells; moreover, in presence of PAX5/ETV6, we observed a slight further increase of H19 (FC=1.67; t test, P=0.0302), mir-675-3p (FC=373.44; t test, P=0.0085) and 5p (FC=24.41; t test, P=0.0001) increased of hundreds time. *Conclusions*. PAX5/ETV6 activates mir-675-3p and -5p and their immature precursor H19 gene, which is physiologically repressed by PAX5. Considering, that microRNAs have a role in normal and pathological hematopoiesis, and that PAX5 regulates oncogenic transcription factors through the mir15a/16 1 cluster, we may hypothesize that PAX5 could regulates candidate microRNAs whose expression could be altered by PAX5/ETV6, with a potential role in leukemogenesis. Further experiments are needed for corroborating this observation.

CO-062

CI-FISH AND SNPS CORRELATE GENOMIC CATEGORIES WITH RISK STRATIFICATION IN CHILDREN WITH T-ALL

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Introduction. As T-ALL leukemogenesis results from diverse molecular lesions affecting genes implicated in cell proliferation and/or survival, self-renewal, cell differentiation, and cell cycle, full genomic characterization is needed to trace the pathogenetic pathways in each T-ALL case and establish the role and contribution (primary vs secondary) of each abnormality. We set up a combined interphase fluorescence in situ hybridization (CI-FISH) assay to investigate aberrations concurring in T-ALL pathogenesis.[1] Single Nucleotide Polymorphisms (SNP) have been largely used in T-ALL to search for genome-wide losses of heterozygosity (LOH) which derive from gene deletions/amplifications and copy number neutral LOH (CNN-LOH) due to somatic recombinations. The present study combines CI-FISH and SNP for the first time to investigate the genomic background of T-ALL in children. *Methods.* We selected 51 children with T-ALL (38 males, 13 females, age range 3-18), who had been enrolled in the AIEOP-BFM ALL2000 protocol. In 50 patients DNA samples at diagnosis and on days +33 and +78 were screened for T-cell receptor rearrangements and classified as standard (MRD-SR, 11 cases), intermediate (MRD-IR, 12 cases) or high risk (MRD-HR, 27 cases).[2] CI-FISH was performed in 40 cases; SNPs in 26. *Results.* CI-FISH was abnormal in 39/40 patients. It detected 2 genetic changes in 27/40. It identified rearrangements of transcription factors (bHLH, LIM; and homeobox), or factors underlying HOXA overexpression in 34 cases: TAL/LMO in 15 cases, HOXA in 9, TLX3 in 8, and TLX1 in 2. Recurrent genomic imbalances were loss of CDKN2A/B deletion (23 cases), GRIK2-CASP8AP2/6q15-16 (4) and LEF1/4q25 (3). SNPs were abnormal in all cas-

es and revealed 121 copy number variations with a mean of 5.7 abnormalities per case. The most common CNV was mono-or bi-allelic CDKN2A/B/9p21 deletion (25 cases); loss of LEF1, CASP8AP2, and PTEN (3 cases each); loss of FBXW7 and RB1 and gain of MYB (2 cases each). Loss of PHF6, IKZF1, LMO2, WT1 and PTPN2, del(1)(p32)/SIL-TAL and del(9)(q34)/SET-NUP214 were found in 1 case each. MRD. Most cases with TAL/LMO and HOXA fell within MRD-HR while TLX3 positive cases were mainly distributed in MRD-SR and MRD-IR groups. The 2 TLX1 positive cases were respectively assigned to MRD-SR and MRD-IR. *Conclusions.* CI-FISH is a promising surrogate of other advanced technologies for classifying pediatric T-ALL into main genomic categories. SNPs provided an in-depth picture of secondary mutations in each genomic category, helping to trace specific leukemic pathways in individual patients. By linking genomic lesions with MRD risk categories a prognostic stratification at diagnosis is provided in ~90% of patients. *Aknowledgments.* FCRP Cod. 2012.0108.021 Ricerca scientifica e tecnologica.

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CO-063

ASXL1 AND GNAS COPY NUMBER VARIATIONS (CNVS) IN MYELOID DISEASES WITH 20Q- OR MONOSOMY 20

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Introduction. Chromosome 20q deletion (20q-) is typically associated with myeloid diseases with a ~6Mb common deleted region (CDR) at bands 20q11.21-q13.13. These bands are the site of the ASXL1 and GNAS genes. In myeloid disorders ASXL1, mapping at 20q11.21, undergoes somatic loss-of-function mutations and deletions while germline mutations cause the Bohring-Opitz syndrome. ASXL1 encodes for a chromatin remodelling protein that is required for HOX gene activation/silencing. GNAS gene, at 20q13.32, is affected by somatic gain-of-function mutations in solid tumors and MDS while germline loss-of-function mutations are found in Pseudohypoparathyroidism. It encodes for adenylyl cyclase stimulatory G (G alpha(s)), a signal transduction protein. This study investigated ASXL1 and GNAS rearrangements in myeloid diseases with 20q-/-20. *Methods.* We retrieved 63 patients with a 20q-/-20 from databases of the Cytogenetic Laboratories, Hematology Departments, Universities of Perugia and Bologna. The 20q-/-20 was isolated in 41 cases and non-isolated in 22 (4 cases with 1 additional change; 18 with a complex karyotype). Diagnoses were: 35 MDS, 11 AML, 9 MPN, 3 MPN/MDS, 3 anemia without morphological evidence of MDS, 1 ALL and 1 Schwachman syndrome. FISH investigated ASXL1/20q11.21 (RP11-358N2) and GNAS/20q13.32 (fosmids G248P80239D1 and G248P80321H8), the CDR (LSI D20S108 20q Vysis/Abbott Molecular) and the subtelomeric region (RP1-81F12). *Results.* FISH confirmed a 20q- in 60/63 patients, identifying 3 types of deletions: interstitial in 56 cases, terminal in 2, intermittent in 1, and intermittent/terminal in 1. All deletions were monoallelic. In 59 cases deletions encompassed locus D20S108/20q12. The exception was 1 AML. ASXL1 copy number variations (CNVs) were found in 18/60 cases (30%). The 3'ASXL1 was partially deleted in 2 MDS. ASXL1 was completely deleted in 14/60 cases (23%) belonging to all disease categories, with isolated 20q- (9/41 cases, 21.9%) and non-isolated 20q-(5/23 cases, 21.7%). ASXL1 gain was found in 1 MDS with non-isolated 20q- and 1 MPN with isolated 20q-. GNAS CNVs were found in 14/60 cases (23.3%). GNAS deletion in 8 MDS, 1 AML and 1 ALL (10/60, 16.6%) was significantly associated with non-isolated 20q- (34.7% vs 4.8% isolated) (Fisher exact test P=0.00); GNAS gain was detected in 3 MDS and 1 MPN with isolated 20q- and in 2 MDS with non-isolated 20q-. *Conclusions.* This study confirmed the heterogeneous size of 20q- deletions, did not identify a CDR and showed GNAS and ASXL1 CNVs are recurrent events in myeloid malignancies. For the first time, GNAS deletion was associated with high-risk karyotype while gain was identified in MDS with isolated 20q-. Interestingly, in 2 MDS a partial 3'ASXL1 deletion suggests ASXL1 underlies gene fusion(s), as observed in B-cell acute lymphoblastic leukemia. *Aknowledgments.* FCRP: 2012.0108.021 ricerca scientifica e tecnologica.

CO-064

OVEREXPRESSION OF WILMS' TUMOR GENE 1 (WT1) IN ACUTE PROMYELOCYTIC LEUKEMIA

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Introduction. WT1 gene is known to be highly expressed in the majority of acute myeloid leukemia (AML), but remains unclear its role in the development of leukemic cells. WT1 gene is note to be expressed in highly proliferative cells (1) and recently, an anti-apoptotic effect of WT1 has been demonstrated in acute promyelocytic leukemia (APL)-cell line (2). However there are only few data regarding expression levels of WT1 in APL. *Patients and Methods.* Between January 2007 and June 2011 we evaluated the WT1 expression in 169 AML patients at diagnosis. To assess the pattern of expression in different subtypes of AMLs, we performed a quantitative polymerase chain reaction (RQ-PCR) assay on bone marrow (BM) samples using the ELN ProfileQuant Kit (Ipsogen, Marseille, France) following the European Leukaemia Net protocol. As normal expression cut-off was established 250 WT1 copies/104 copies Abelson (ABL). According to WHO classification of myeloid neoplasm we observed: 49 (29%) AMLs with myelodysplasia–related changes, 27 (16%) AMLs with minimal differentiation, 15 (9%) AMLs with maturation, 22 (13%) Acute Myelomonocytic leukemia, 35 (21%) Acute Monocytic leukemia and 21 (12%) APL. All the APL were PML-RARalpha and t(15;17)(q22;q12) positives. *Results.* There were only 9 WT1 normal expressing patients (5%), whereas 160 (95%) AML cases presented levels of WT1 expression higher than the established cut-off, with a median value of 6776 copies WT1/104 copies ABL (range 235-62567). Inside each FAB subtypes, the median level of WT1 in the overexpressing group at diagnosis was: 8669 WT1 copies/104 copies ABL (range 1368-30529) in M0-M1 group, 4995 copies (1008-25585) in M2 group, 30110 copies (2069-62597) in M3 group, 8111 copies (235-41148) in M4 group, 6226 copies (416-15661) in M5 group, 4530 copies (353-24205) in secondary AML (Msec) group. The level of WT1 expression in APL was significantly higher (P<0.01) than in all other subtypes (Figure 1). All APL patients presented an overexpression of WT1, differently from M4, M5 and secondary AML groups. Moreover, the majority of APL patients (81%, 17/21 pts) had more than 20000 WT1 copies at onset. Conversely, only 9 out of 139 (6%) Non APL cases demonstrated a number of WT1 copies higher than 20000.

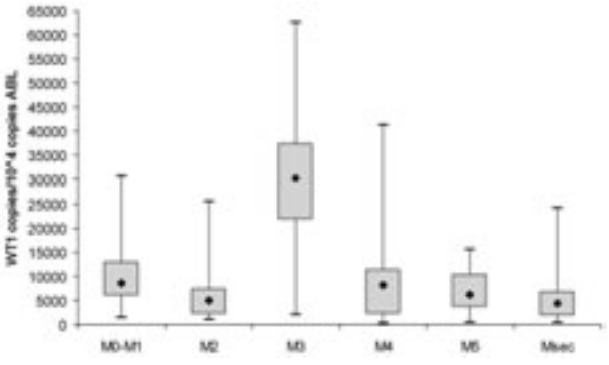

Figure 1. Absolute quantification of WT1 gene in different AML FAB subtypes.

Conclusions. In our cohort of AML patients, Acute Promyelocytic Leukemia group showed the highest levels of WT1 expression. In particular, we observed that all APL patients overexpressed WT1 gene at diagnosis and the transcript levels were significantly higher in APL cases than in other FAB subtypes. It is still unclear the role of WT1 gene expression to promote or sustain proliferation of leukemic cells, but this observation could reinforce a possible relationship between the pathway stimulated by PML-RAR transcript and the WT1 anti-apoptotic and pro-proliferative mechanisms.[1,2]

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Monoclonal Gammopathies

CO-065

IDENTIFICATION OF MYELOMA-ASSOCIATED MIRNA AND THEIR PROGNOSTIC VALUE: A COMPENDIUM FROM UK MRC MYELOMA IX TRIAL

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Introduction. MicroRNAs (miRNAs), small noncoding RNAs involved in a several healthy or pathological cellular processes, are emerging as crucial player in multiple myeloma (MM). *Methods*. We performed integrative analyses of gene and miRNA expression and genome-wide profilings of 163 primary tumors included in the UK Myeloma IX clinical trial. Global miRNA profiles were generated on GeneChip® miRNA arrays (probing 847 human mature miRNAs); expression values were extracted with Affymetrix miRNA QC Tool and RMA-normalized. The miRNA profiling data were combined with the transcriptional profiles of 152 and the genotyping data of 50 matching samples, generated on GeneChip® HG-U133Plus2.0 and Human Mapping 500K arrays, respectively. The transcriptional data were generated using GeneAnnot-based annotations and RMA normalization; the copy number (CN) values were inferred against normal germ-line counterpart for each sample using circular binary segmentation and FISH-based normalization procedures in R/Bioconductor. *Results*. Correlation analysis revealed 58 different mature miRNA transcripts whose expression levels were concordant with the inferred CN of their miRNA genes. A large fraction of the identified miRNAs mapped to chromosome 1 (19%); the expression of mir-19a, mir-19b and mir-20a, included in mir17~92 cluster, correlated with the CN of their locus at 13q31.3. The analysis of miRNA expression profiles in the context of the main MM molecular subtypes mostly confirmed that t(4;14) patients showed specific overexpression of let-7e, miR-125a-5p, and miR-99b (clustered at 19q13.33), as previously shown by us in a smaller cohort (Lionetti et al, 2009). Moreover, we integrated computational miRNA target prediction with miRNA/gene expression profilings, leading to define putative functional miRNA-target regulatory relationships, supported by expression data and at least 3 of 9 target computation algorithms. After multiple test correction, 13 highly significant relationships emerged, among which 6 (let-7e with THG1L, SLC2A8 and C11orf57, miR-125a-5p with RPL28 and C11orf57, and miR-99b with RPL28) involved the miRNAs deregulated in t(4;14) cases. The evaluation of miRNA expression in association with clinical data indicated that 5 miRNA (miR-17, miR-18a, miR-106a, miR-21, and miR-206) strongly correlated with overall survival in MM, either per se or in concerted fashion. In this way, we defined a 2-miRNA model able to stratify MM into 4 significantly different prognostic subgroup (P=2E-05), independent of the main cytogenetic alterations [t(4;14) and t(11;14) translocations, 1q gain, 13q and 17p deletions, hyperdiploidy], ISS stage (and each serum 2-microglobulin and albumin level) and Myeloma IX cases treatment arm. *Conclusion*. Overall, our integrated analysis on a comprehensive dataset from controlled clinical trial added insights for further functional assessment of miRNA role in MM biology, and highlighted likely miRNA involvement in MM outcome.

CO-066

GENOME-WIDE APPROACH IDENTIFY RECURRENT 8P21.2 LOSS IN MORE AGGRESSIVE FORM OF PRIMARY PLASMA-CELL LEUKEMIA

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Introduction. Plasma cell leukemia (PCL) is a rare and aggressive form of plasma cell (PC) neoplasm, which can originate *de novo* (primary, pPCL) or progress from pre-existing Multiple Myeloma (secondary, sPCL). PCL is characterized by a marked genomic instability and, to date, genome-wide studies are still limited. We aimed at identify novel molecular lesions and their possible association with a distinct gene-expression pattern. *Methods.* Highly purified (≥90%) bone marrow PCs samples were obtained from 23 newly-diagnosed pPCL patients, enrolled in a multicenter GIMEMA clinical trial testing the lenalidomide/low dose dexamethasone combination in first-line treatment of pPCL. All the samples were characterized, by FISH, for the main IGH chromosomal translocations, 13q14, 17p13, 1p33 (CDKN2C) deletions and gain at 1q21.3 (CKS1B). Based on our previously described FISH standard protocols (Fabris *et al.*, 2005), specific BAC clone RP11-795G8 for 8p21.2 was selected to validate SNP-array data. Genome-wide DNA profiles were obtained using the Affymetrix GeneChip® Human Mapping 250K Nsp and copy number values were inferred through circularly binary segmentation and FISH-based normalization procedures. Gene expression profiles were generated on GeneChip® Gene 1.0 ST Array. *Results.* Alterations of chromosome 8 were observed in 10/17 patients (58.8%) analyzed by SNP-array. In particular, extensive 8p monoallelic deletions were observed in 6 cases whereas a homozygous deletion at 8p21.2, defining a putative minimal altered region (MAR) containing the PPP2R2A gene, was detected in one patient. SNP-array data of 13 patients were confirmed by FISH in all but one case showing an additional biallelic deletion in only 10% of cells, not identified by SNP analysis. Furthermore, among two additional cases analyzed only by FISH, one case showed a monoallelic deletion. Overall, the putative MAR at 8p21.2 was lost in 8/19 (42%) patients. Finally, a supervised analysis was made between the 8p deleted (8 pts) and 8p normal (10 pts) pPCLs analyzed by Gene 1.0 ST Array. Eight genes were found down-regulated in pPCLs with 8p loss, two of which mapped on 12q and six at 8p (4 in 8p21.1-8p21.3 and 2 in 8p12). In addition to PPP2R2A gene, implicated in the negative control of cell growth and division, we evidenced others potentially relevant genes, involved in positive regulation of apoptosis (KIAA1967 and TNFRSF10B), DNA repair, replication and telomere maintenance (WRN) and in translation machinery (MAK16). *Conclusions.* Our combined genomic and gene expression profiles approach reveal a gene-dosage effect associated to 8p loss providing insight for defining novel candidate disease-associated genes.

CO-067

COMPROMISED ACTIVITY OF NUCLEAR SIRTUINS SENSITIZES BRCANESS MULTIPLE MYELOMA CELLS TO DNA DAMAGE AGENTS

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Background. Multiple myeloma (MM) is a clonal malignancy of plasma cells with a striking genetic instability. This represents a very harmful disease process fundamental to the invasion and progression of MM cells. Consequently, inhibition of DNA repair mechanisms leads to significant reduction in acquisition of new genetic changes as well as progression of MM. Mammalian sirtuins are class III NAD+-dependent histone deacetylase emerging as innovative proteins involved in multiple pathways, including genome maintenance. *Methods.* a panel of 18 different MM cell lines, both sensitive and resistant to conventional

and novel anti-myeloma drugs, was used in the study. The antitumor effect of Nicotinamide combined with chemotherapic agents was investigated by CTG assay and Annexin-V/propidium iodide staining. Mechanistic studies were performed with thymidine incorporation, Western-blotting, lentivirus-mediated shRNAs and immunofluorescence. Finally, analysis of DNA DSB repair by chromosomally integrated reporter constructs followed by cytometer analysis was carried out. *Results.* We analyzed an Affymetrix GeneChip (GSE6477) for MM primary tumors (n=162) and normal plasma cells founding that transcript levels of two nuclear sirtuins (SIRT6 and SIRT7) were significantly higher in MGUS, smoldering myeloma, MM and relapsed cases when compared to normal donors. Importantly, a protein analysis assay confirmed increased levels of both sirtuins in 18 different MM established cell lines, including those resistant to novel (ANBL6-BR) and conventional (MM.1R, LR-5, Dox40) therapeutic agents, compared to PBMCs of healthy donors. Next we evaluated the functional role of Sirt6 and 7 in MM cells by using loss of function approaches with RNAi. As SIRT6 and SIRT7 silencing reduced MM cell proliferation compared with control scrambled cells but did show only a slight induction of cytotoxicity. We also examined the effects of Nicotinamide (Nam), a pan-sirtuins inhibitor, on DNA damage response signaling triggered by conventional anti-MM agents (Melphalan, Doxorubicin and 5-AZA). Nam treatment did not appreciably affect MM cell viability, conversely Nam pretreatment impaired DNA double-strand breaks (DSBs) as well as DNA repair mechanisms triggered by conventional DNA damage agents, as documented by H2AX and RPA phosphorylation, respectively. Consistent with these findings, Nam-pretreated cells not only were impaired in forming RAD51 foci in response to Doxorubicin and Melphalan, but also caused their hypersensitivity. Importantly, this sensitizing effect was observed also in MM cells selected for resistance to Doxorubicin (RPMI-Doxo40) or Melphalan (LR5), indicating that Nam increases chemosensitivity in both drug-sensitive and –resistant MM cells. Similarly, lentivirus-mediated shRNA interference SIRT6 and 7 depletion sensitized cells to Melphalan and Doxorubicin. Finally, by using MM cell lines containing chromosomally integrated green fluorescent protein-based reporter constructs, chemical as well as genetic approaches improved the efficiency of both HR and NHEJ. Ongoing in vivo experiments are assessing how the chemical susceptibility of SIRT6 and/or 7-deficient cells can be exploited therapeutically. *Conclusion.* Our study strongly points towards the existence of a link between nuclear sirtuins and DNA instability of MM cells, providing the basis for further and innovative anti-MM therapeutic approach.

CO-068

INTERLEUKIN-3 INDUCES ACTIVIN A IN BONE MARROW MONOCYTES: ROLE IN MULTIPLE MYELOMA PATIENTS

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Introduction. Interleukin-3 (IL-3) is a multifunctional cytokine that indirectly increases osteoclastogenesis and suppresses osteoblastogenesis via CD14+ bone marrow (BM) monocytes. IL-3 levels are elevated in the BM plasma of MM patients compared with healthy controls suggesting a pathopyisiological role of this cytokine in multiple myeloma (MM). However, the mediators of IL-3's effects on MM bone disease are unknown. *Methods and Results.* To investigate this issue we performed gene expression profiling using Affymetrix GeneChip® analysis of IL-3 treated BM monocytes from MM patients and found ActA gene expression was increased 180-fold and confirmed this finding at the protein level by ELISA. ActA is a negative regulator of bone mass that promotes osteoclastogenesis and is overproduced in MM patients. Interestingly we found that ActA was produced by MM and MGUS patient CD14+ cells treated with IL-3 to a significantly higher degree compared to healthy subjects. (Median ActA levels for MM and MGUS ActA levels were increased 66.57 and 51.6 fold respectively over untreated cells, while IL-3 treatment of normal cells increased ActA 8.5 fold.) ActA levels were also increased in freshly isolated marrow plasma of a cohort of patients with symptomatic MM (n=96) as compared to patients with smoldering MM (SMM) (n=22), MGUS (n=31), or healthy subjects (n=10) (median ActA levels: active MM 486 pg/mL, SMM 395 pg/mL, MGUS 361 pg/mL, and healthy donors 286 pg/mL). The potential role of ActA in IL-3 mediated osteoclast (OCL) formation was also examined. Culture of BM monocytes with ActA or IL-3 significantly enhanced osteoclastogenesis compared with control and ActA enhanced RANKL-induced osteoclastogenesis. Osteoprotegerin treatment of normal donor BM monocytes stimulated with ActA failed to block the osteoclastogenic effects of ActA, demonstrating that ActA's osteoclastogenic effects were RANKL independent. Importantly, the osteoclastogenic effect of IL-3 was dose-dependently inhibited by anti-ActA, and IL-3 induced ActA expression by BM monocytes decreased during OCL differentiation. In support of early OCL precursors as the source of IL-3 induced ActA, we did not identify IL-3 receptors on mature OCL by flow cytometry. *Conclusions.* These results demonstrate that IL-3 induction of osteoclastogenesis is mediated by ActA produced by CD14+ BMM and is RANKL independent. Thus, we hypothesize that therapies targeting the ActA receptor, such as the recently developed ActA receptor antagonist, should block both IL-3 and ActA, and thereby significantly impact MM bone disease via their effects on BM monocytes.

CO-069

RESISTANCE OF MULTIPLE MYELOMA CELLS TO TRAIL IS OVERCOME BY BLOCKADE OF AURORA KINASES

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Introduction. Recombinant human (TNF)-related apoptosis-inducing ligand (TRAIL) ligand and agonistic mAbs have shown remarkable promise as an anticancer agent. However, TRAIL signaling also activates survival signaling that can compromise its efficacy and strategies have been developed to bypass TRAIL resistance in diverse preclinical models and are based on the combination of TRAIL and numerous conventional and investigational anticancer agents. The aim of this study was to investigate whether the combined treatment with pan-Aurora kinase inhibitors (pan-AKIs) and TRAIL has cytotoxic effects on Multiple Myeloma (MM) cells. *Methods.* The anti-myeloma activity of Pan-AKIs plus TRAIL was evaluated *in vitro* using MM cell lines or patient-derived MM cells, and in vivo in a human plasmacytoma xenograft mouse model. In the *in vitro* experiments apoptotic ratio was measured by flow cytometry; expression, activation and function of signaling proteins were analyzed by Western blotting and SiRNA technologies; Survival, tumor growth inhibition, histology, Immunohistochemistry and western blotting were performed to evaluate the anti-myeloma activity of Pan-AKIs/TRAIL in vivo. *Results.* We demonstrate that targeting Aurora A and B kinases with selective pan-AKIs strikingly enhances TRAIL-induced cytotoxicity in TRAIL-sensitive and -resistant MM cell lines, as well as patient-derived MM cells through a caspase-dependent mechanism. Co-treatment with pan-AKIs and TRAIL strikingly elevates the (DR4+DR5)/(DcR1+DcR2) TRAIL receptors ratio, enhances proapoptotic protein Bak and decreases protein levels of cellular inhibitor-of-apoptosis protein 1 (c-IAP1), c-IAP2 and X chromosome-linked IAP (XIAP). Furthermore we found that the combination of pan-AKIs with TRAIL reduced the basal and/or TRAIL-induced expression of the anti-apoptotic Bfl-1/A1 and Mcl-1, both relevant targets for TRAIL sensitization in MM cells. To assess the in vivo efficacy of combining TRAIL and Pan-AKIs, we tested these compounds using a drug-resistant human plasmacytoma xenograft mouse model. Importantly, when Pan-Aurora inhibitors were combined with TRAIL, there was a significant ($P < .001$ Dunnet test) reduction in tumor growth relative to either treatment alone. Furthermore, the combination pan-AKIs/TRAIL significantly ($P < .001$; Kaplan-Meier method and compared using the log-rank test, followed by a Bonferroni correction for multiple comparisons) prolonged survival compared with treatment with either drug alone and was well tolerated in vivo; the histopathological, immunohistochemical and western blotting analysis of tumors revealed a stronger activation of caspase-3 and PARP fragmentation in tumors from Aurora inhibitors plus TRAIL-treated mice relative to either treatment alone. *Conclusion.* These findings suggest that combining pan-AKIs with TRAIL may have therapeutic benefit in MM.

CO-070

MYELOID IMPAIRMENT CONTRIBUTES TO IMMUNOPARESIS IN PROGRESSION OF MULTIPLE MYELOMA THROUGH MGUS

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Introduction. In Multiple Myeloma (MM), but not in the monoclonal gammopathy of unknown significance (MGUS), the immune function is impaired as consequence of an immunologically hostile microenvironment and cellular defects, including reduction of immuno-surveillance and T-cell immunoparesis. We conducted an extensive immunological study focused on determining the relationship between the myeloid and lymphoid impairment in MM, and its role in the progression from MGUS to MM. *Methods.* Between January 2009 and April 2011 peripheral blood obtained from 60 consecutive newly diagnosed MM and 70 MGUS plus 30 healthy subjects was studied for evaluation of myeloid subpopulations and lymphoid paresis. We identified immature im-MDSC as CD11b+, CD13+, CD14-, CD34+,HLA-DR-, CD45+ cells, neutrophilic-like N-MDSC as CD11b+, CD13+, CD15+, CD14-, HLA-DR-, Lin- cells and monocytic-like mo-MDSC as CD14+, HLA-DRlow/-. Myeloid function was evaluated by phagocytic activity using a commercially available kit (Phagotest R). Lymphoid subpopulations were evaluated as absolute number of circulating CD200+/CD3+ tolerogenic T cells, CD4+CD25+FoxP3+ T-reg and expression of CD62L (normalised mean of fluorescence intensity, N-MFI). *Results.* The capability of phagocytosis of in neutrophils and monocytes from MM patients at diagnosis was significantly reduced compared to healthy subjects (P<0.001) and MGUS (P<0.0001). Both mature suppressive N-MDSC and mo-MDSC subsets were not significantly increased in MGUS and MM patients, even if mo-MDSC showed an increasing trend from healthy donors through MM (P=0.06). On the opposite, im-MDSC subset was higher in MM vs healthy (P=0.002) and MGUS (P=0.001). Treg were reduced in MM patients compared to healthy subjects (P<0.0001), while MGUS showed absolute count with an intermediate level between MM and healthy controls. im-MDSC absolute count was negatively correlated to Treg absolute count (R=0.37, P<0.001). Mature subset of N-MDSC and mo-MDSC were negatively correlated to levels of CD62L-N-MFI on CD8+ cells (respectively, R=0.33, P=0.006 and R=0.28, P=0.002). 30/60 MM patients undergoing to treatment with bortezomib- or lenalidomide-based regimens were evaluable for response after therapy, with a follow up >6 months. After the induction therapy, patients with at least partial remission showed normalization of im-MDSC and mo-MDSC levels, independently from the drug combination used. *Conclusions.* Taken together, our findings suggest that in MM but not in MGUS there is a myeloid cell dysfunction that is correlated to impairment of T- cell arm. These alterations may have a role in the progression from MGUS to MM.

CO-071

A 41-GENE SIGNATURE PREDICTS COMPLETE RESPONSE (CR) TO BORTEZOMIB-THALIDOMIDE-DEXAMETHASONE (VTD) AS INDUCTION THERAPY PRIOR TO AUTOLOGOUS STEM-CELL TRANSPLANTATION (ASCT) IN MULTIPLE MYELOMA (MM)

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Background. Achievement of CR is generally associated with improved clinical outcomes for patients (pts) with MM and represents a primary endpoint of current clinical trials. The GIMEMA Italian Myeloma Network phase 3 study demonstrated that VTD regimen was superior over thalidomide-dexamethasone (TD) as induction therapy prior to double ASCT for newly diagnosed MM (rate of >nCR: 31% for the 236 pts on VTD, 11% (P<0.0001) for the 238 pts on TD). Since enhanced rates of >nCR affected by VTD resulted in extended progression-free survival, prediction of CR by pharmacogenomic tools is likely to be an important goal to prospectively select those pts who are more likely to benefit from a given therapy. *Methods.* We assessed the ability of GEP to predict attainment of CR in 122 pts enrolled in the VTD arm of the study. CD138+ plasma cells were obtained at diagnosis from each pts and were profiled for gene expression (Affymetrix U133 Plus2.0 platform). *Results.* 34/122 pts (28%) who were included in the present analysis achieved a >nCR. Several gene signatures were identified, among which we choose a 163-gene signature that provided a predictive capability of 79% sensitivity, 87% specificity, 71% positive predictive value (PPV) and 92% negative predictive value (NPV). These expression values were used in an unsupervised hierarchical clustering to stratify the population of 122 profilated pts into 3 subgroups (subgroup A, 79 pts; subgroup B, 22 pts; subgroup C, 21 pts). Notably, 19/34 >nCR pts (56%) clustered in subgroup B, whereas the remaining 15 pts were randomly distributed within subgroup A. Analysis of disease characteristics revealed that in subgroup B the frequencies of pts carrying del(13q) (78%) or del(17p) (22%) or with an IgA isotype (54%) were significantly higher in comparison with the corresponding values found in subgroup A (47%, 4%, and 10%, respectively) and subgroup C (38%, 10%, and 5%, respectively). In order to obtain a more feasible set of genes predictive of >nCR, several smaller signatures originating from the 163-gene signature were further analyzed. The best predictive capability was obtained with a 41-gene signature that provided 88% sensitivity, 97% specificity, 91% PPV and 95% NPV. A GeneGo® network analysis showed that the most relevant network nodes included tumour suppressor genes (FBXW7 and MAD), genes involved in inflammatory response (TREM1 and TLR4) and genes involved in B cell development (IKZF1, IL10 and NFAM1). *Conclusions.* GEP analysis of a subgroup of pts who received VTD induction therapy allowed to provide a 41-gene signature that was able to predict attainment of >nCR and, conversely, failure to achieve at least nCR in 91% and 95% of cases, respectively. These favorable results might represent a first step towards the possible application of a tailored therapy based on the single patient's genetic background. *Supported by.* Fondazione Del Monte di Bologna e Ravenna, Ateneo RFO grants (M.C.) BolognAIL.

CO-072

TRANSCRIPTOME ANALYSIS OF PRIMARY PLASMA CELL LEUKEMIA TUMORS FROM A MULTICENTER PROSPECTIVE GIMEMA STUDY: BIOLOGICAL AND CLINICAL IMPLICATIONS

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Introduction. Plasma cell leukaemia is a rare aggressive disease which may originate *de novo* as primary (pPCL) or in the context of a pre-existing multiple myeloma (MM) as secondary PCL (sPCL). To date, global transcriptomics studies in pPCL are still limited. *Methods.* The transcriptional profiles of highly purified plasma cells (PC) from 21 previously untreated pPCL, enrolled in an Italian GIMEMA multicenter clinical trial, and 55 MM patients were generated on GeneChip® HuGene 1.0 ST array. Conventional agglomerative clustering was used to test the natural grouping of samples. Supervised analysis using samr package in R environment was applied to find the genes specifically modulated in pPCLs versus MMs. All samples were characterized for major chromosomal aberrations by FISH analysis. An independent publicly available dataset of 300 samples profiled on GeneChip® HG-U133A array, including 18 healthy donor (N), 28 Monoclonal Gammopathy of Undetermined Significance (MGUS), 19 smoldering MM (SMM), 200 newly-diagnosed and 26 relapsed MM (RMM), and 9 sPCL cases, were used to test the progressive modulation of the identified transcripts from normal condition along the different forms of PC dyscrasia. Globaltest and survival packages for Bioconductor were used to investigate the correlation of expression data with outcome. *Results.* Unsupervised analyses of the most variable genes in pPCL revealed that sample grouping was mainly driven by the major IgH chromosomal translocations, as observed in MM. Supervised analysis between the 21 pPCL and 55 MM samples identified 366 up- and 137 down-regulated genes in pPCL, significantly enriched in functions related to immune system development, apoptosis, cytoskeleton organization and cell migration. Of these genes, in an extended dataset encompassing the progressively malignant stages of myeloma (N, MGUS, SMM, MM, RMM, sPCL), we reported that 28 were also modulated in the transition from N through sPCL, most of them (19/28, 68%) showing a significant decrease in the trend of expression levels. Among them were genes of proteasome complex (PSMA7, PSMD6), cytoskeleton and extracellular matrix (C13orf15, CRISP3, MMP8), involved in cell adhesion (CD36, SLAMF7, SSPN, VCAM1) and cell migration (CORO1A, RAP2C). The evaluation of outcome revealed that the occurrence of the major cytogenetic aberrations did not affect survival. However, we identified a 38-gene signature which clearly clustered those cases with poorest prognosis [P=.001, hazard ratio = 9.41 (1.79-49.33 95% CI)] and whose significance in multivariate analysis retained independence from cytogenetic alterations. *Conclusions.* The comprehensive transcriptional analysis of pPCLs included in a prospective clinical trial allowed to outline genes that characterize aggressive forms, as well as to define a gene signature able to discriminate high-risk pPCL.

Lymphomas

CO-073

FINAL RESULTS OF A MULTICENTRE TRIAL ADDRESSING ROLE OF CSF FLOW CYTOMETRY ANALYSIS IN NHL AT HIGH RISK FOR CNS DISSEMINATION

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Introduction. The overall incidence of central nervous system (CNS) dissemination in patients with aggressive non-Hodgkin Lymphoma (NHL) is about 5%. Prophylactic treatment likely reduces the incidence of CNS relapse but may increases the toxicity of systemic chemotherapy. Therefore, the identification of patient subgroups for which CNS prophylaxis may be useful is important. This prospective study was aimed to compare diagnostic and prognostic value of conventional cytologic (CC) examination and flow cytometry (FCM) of cerebrospinal fluid (CSF) in a large cohort of patients with newly diagnosed aggressive B-cell lymphoma at high risk for CNS dissemination. *Methods.* Selection criteria were: diagnosis of aggressive B-NHL with increased risk for CNS dissemination (lymphoblastic, Burkitt, blastoid variant of mantle cell, or diffuse large B-cell with involvement of testes, base of the skull, epidural structures and bone marrow, or age-adjusted IPI score 2-3 with involvement of >one extranodal organs and increased LDH); no signs of neurological disease; planned standard intrathecal prophylactic therapy. Multiparameter analysis was based on antigen expression and morphological properties defined by forward and side scatter. *Results.* One-hundred seventy-four patients were enrolled in 12 Italian centres. FCM detected a neoplastic population in the CSF of 18 out of 174 patients (10%), CC only in 7 (4%) (P<0.001); 11 patients (14%) were discordant (FCM+/CC-). At a median follow up of 46 months, there were 64 systemic progressions, 10 CNS relapses, included two patients wit both systemic and CNS relapse. Two-year PFS and OS were significantly higher in patients with FCM-negative CSF (62% and 72%) compared with those FCM-positive CSF (39% and 50%) with a 2-year CNS relapse cumulative incidence of 17%(95%CI:0-34) vs. 3%(95%CI:0-7)(P= 0.004), respectively. Discordant patients showed a significant increased risk of CNS progression at 2-year respect to both negative patients (HR 8.16, CI95%:1.45-46). *Conclusion.* FCM positivity in CSF of patients with high-risk NHL is associated with a significantly higher CNS relapse risk and poorer outcome. Brain parenchyma was the commonest site of CNS failure, suggesting that the combination of i.v drugs with a higher CNS bioavailability and intrathecal chemotherapy is advisable to prevent CNS relapses in FCM+ patients. Registered at *http://www.clinicaltrials.gov: NCT00949741.*

CO-074

THE SMALL MOLECULE CHK1/CHK2 INHIBITOR PF-0477736 (PFIZER) DEMONSTRATES SINGLE AGENT ACTIVITY IN PRECLINICAL MODELS OF DIFFUSE LARGE B-CELL LYMPHOMA

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Introduction. Few data are available on the role of CHK inhibitors in Diffuse Large B cell Lymphoma (DLBCL). The checkpoint kinases 1 (CHK1) and 2 (CHK2) are serine-threonine kinases involved in the DNA damage response pathway. CHK inhibition enhances the efficacy of DNA damaging agents in a variety of tumors, including p53 deficient cells, that rely on the G2/M checkpoint. DLBCL with dysfunctional p53 axis (harboring p53 mutations and/or CDKN2A loss) have been recently shown to have a dismal outcome. In this study we report the activity profile of the CHK1/2 inhibitor PF-0477736 (Pfizer) in a panel of B cell lymphoma cell lines and primary cells. *Methods.* Three Germinal center B cell (GCB) DLBCL derived cell lines (SUDHL-4, SHDHL-6, BJAB), 3 Activated B cell (ABC) DLBCL (HBL-1, U2932, TMD8), one mantle cell lymphoma (Mino), and the Hodgkin Lymphoma cell line KM-H2 were first screened for p53 and CDKN2A mutations and deletions. All amplicons were sequenced by conventional Sanger method. Cell viability was assessed by WST-1 assay (Roche). *Results.* P53 mutations were detected in the following cell lines: HBL-1, U2932, SUDHL-6, BJAB, Mino. TMD8 was p53 wild-type but with an homozygous deletion of CDKN2A. SUDHL-4 and KM-H2 were p53 wild type, with no deletion of CDKN2A. To assess the effect of PF-0477736 on cell proliferation, cells were incubated with increasing concentrations of PF-0477736 (from 5 to 2000 nM) for 24, 48 and 72 hours (hrs). A significant growth inhibition was evident after 48 hrs, in all cell lines (IC50 140 to 230 nM), excluding KM-H2 cells that were resistant (IC50 6800 nM at 48 hrs). Using Annexin V-propidium iodide staining, we found that PF-0477736 25-500 nM induced cell death by apoptosis in a time and dose dependent manner. PF-0477736 100-1000nM demonstrated activity also in primary DLBCL cells. Of note no significant cytotoxicity was observed in hematopoietic progenitors from bone marrow aspirations of lymphoma patients without bone marrow involvement. We found no correlations between baseline levels of CHK1/2 activation and outcome. In the sensitive cell lines inhibition of the downstream target CDC25c ser216 phosphorylation coupled with a marked increase in levels of the DNA damage marker H2AX was observed by western blot as soon as after 24 hrs of incubation with concentrations equal to the IC50 (25 – 250 nM). In the resistant KM-H2 cell line no increase in levels of H2AX was observed. PF-0477736 at the dose of 50-100 nM synergistically enhanced the efficacy of Doxorubicin (0.1 to 1 M) at 24 hrs. *Conclusions.* These data suggest that PF-0477736 has single agent activity and synergizes with chemotherapy in DLBCL. The drug shows high single agent activity in the subset of DLBCL with genomic lesions of the p53 pathway, that are resistant to conventional chemotherapy and associated with dismal outcome, providing the rationale for further clinical investigation of PF-0477736 in DLBCL.

CO-075

A CASE-CONTROL STUDY OF OCCUPATION, SOLVENT EXPOSURE AND THE RISK OF NON-HODGKIN'S LYMPHOMA

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Introduction Previous epidemiologic findings suggest an association between occupational solvent exposure and non-Hodgkin's lymphoma (NHL). Benzene is a carcinogenic in humans (group 1, IARC); trichloroethylene is classified as probably carcinogenic (group 2b); carbon tetrachloride, used as a solvent, is related to an excess risk of NHL in women; dibromoethane is listed as a carcinogen by the IARC. *Aim.* In our study we performed a population-based case-control study using detailed exposure assessment methods to investigate the association between occupation, solvent exposure and NHL. *Methods.* 109 lymphoma cases and 43 healthy controls have been recruited by the research units of Bari and Taranto within the Italian GxE case-control study, since October 2008. This study, still in progress, participates in the INTERLYMPH GWAS study (International Genome Wide Study). The main purpose of the Interlymph Consortium is to define causal models of lymphoma, using an interdisciplinary approach applying genomics, molecular biology and epidemiological techniques. A 40 ml blood sample was withdrawn from cases and controls, who also underwent an interview using a standardized questionnaire enquiring about occupational histories and workplace exposure to solvents. We conducted a preliminary data analysis to obtain a frequency distribution of the different subtypes of lymphoma recruited so far at our research unit and to carry out a preliminary assessment of the role of the occupational factors using the CAREX matrix. *Results.* We observed a higher risk of lymphoma among blue collar workers at multivariate analysis (OR:4.06 (1.03-15.96). Diffuse large B- cell lymphoma (DLBCL) and Follicular lymphoma (FL), according to the WHO classification, accounted for the majority of lymphoma subtypes. At univariate analysis a significant increase of cancer risk for solvent exposure (benzene, carbon tetrachloride, ethylene dichromate, dibromoethane) was observed (OR:2.24 (1.04-4.97). Moreover, in a multiple unconditional regression model the exposure to carcinogenic solvents, adjusted by several variables, resulted associated to lymphoma (OR:4.06 (1.03-15.96). *Conclusions.* Our preliminary results suggest a higher risk of lymphoma among blue collar workers and an association between occupational exposure to solvents and the risk of NHL. These data will be factored into the INTERLYMPH GWAS study and may help, in a population-based case-control study, and thanks to a consistent methodology and statistical power, to probe the role of occupational and environmental factors in etiological hypotheses, and then devise preventive, diagnostic and therapeutic approaches.

CO-076

EXPRESSION AND FUNCTION OF PROTEIN KINASE CK2 IN MANTLE CELL LYMPHOMA

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Introduction. Mantle Cell lymphoma (MCL) B lymphocytes are characterized by specific genetic alterations, which affect cellular proliferation, survival and response to exogenous and endogenous stresses. MCL, together with multiple myeloma, represents the second hematologic malignancy for which proteasome inhibitor bortezomib has been approved for clinical use in the relapsed setting. We previously showed that protein kinase CK2 is highly expressed in and sustains multiple myeloma cell survival upon different cellular stresses. CK2 is important for the growth of other B-cell malignancies, such as acute lymphoblastic leukemia and chronic lymphocytic leukemia. Since CK2 regulates cellular processes, which are altered in MCL, such as cell cycle, cell survival and stress responses, here we investigated the functional significance of CK2 expression and activity in MCL cells. We determined the effects of CK2 inhibition on bortezomib-induced MCL cell apoptosis. We also analyzed apoptosis-related Bcl2-family members alterations and the NF-kappaB pro-survival signalling pathway, which could lend MCL cells the capability of resisting to bortezomib-induced cytotoxicity. *Methods.* Bortezomib-sensitive (Jeko-1 and Granta-519) and bortezomib-resistant (Rec-1) MCL cell lines as well as freshly isolated peripheral blood CD19+ B lymphocytes from healthy donors and CD19+ CD5+ B lymphocytes from peripheral blood or bone marrow of MCL patients were cultured as per standard methods and exposed to different doses of two CK2 inhibitors (K27 and CX4945) and bortezomib. Cell proliferation and apoptosis were measured by [3H]-thymidine incorporation assay, annexin V and propidium iodide staining, evaluation of mitochondrial potential depolarization and FACS analysis, western blotting of PARP cleavage and apoptosis-related proteins. The combination index of CK2 inhibition plus bortezomib treatment was calcu-

lated. Survival signaling pathways were studied with western blot analysis. *Results.* High levels of CK2 protein were found in MCL cells. CK2 inhibitors had a strong dose-dependent cytotoxic effect on MCL B cells. A synergic cytotoxicity was found by treating MCL cells with bortezomib plus CK2 inhibitors (combination index <1, P<0.05) both in bortezomib-sensitive and -resistant cell lines and in MCL B lymphocytes obtained from patients. Remarkably, normal CD19+ B lymphocytes were not affected by the combined treatments. CK2 inhibitors and bortezomib, caused a reduction of Bcl2 and a parallel increase of Bak and Bax, remarkably mostly in the bortezomib-resistant cell line. Moreover, this treatment treatment led to a marked reduction of phosphorylated NF-kappa B p65 protein. *Conclusions.* Protein kinase CK2 is crucial for MCL B lymphocyte survival and affects the sensitivity to proteasome inhibitors. CK2 could control the threshold of MCL entry into the apoptotic program upon bortezomib treatment and could regulate the activation of an anti-apoptotic signalling dependent on Bcl2 and NF-kappa B. The assessment of the clinical and prognostic role of this protein kinase in MCL is ongoing. Our data suggest that CK2 inhibitors could represent a valuable therapeutic option in the treatment of MCL.

CO-077

EFFECTS OF CHLORAMBUCIL/HYDROXYCHLOROQUINE-LOADED ANTI-CD20 NANOPARTICLES ON A HUMAN/MOUSE MODEL OF BURKITT LYMPHOMA

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Introduction. In Burkitt lymphoma (BL) chemotherapy shows variable responses since a fraction of younger patients are resistant or relapse and the patients over 40 have difficulties to follow the therapy. In non-responders/relapsers BL cells often express low level of CD20 and/or mutant/deleted p53. To overcome these problems, we developed a new therapeutic approach in which the efficacy of chemotherapy is associated to the specificity and low side-effects of antibody-based therapy. This approach lies in biodegradable nanoparticles (NP) coated with an antibody to target the neoplastic cells and filled with Hydroxychloroquine (HCQ) (a p53-independent toxic substance) and chlorambucil (CLB) as modern "Trojan Horses" to destroy the cancer cells. *Methods.* NP were prepared by the copolymerization of polylactic acid and polycaprolactone (diameter:250nm). The NP with HCQ and CLB were prepared by encapsulation at concentrations of 165 µg per mg of polymer inside their core, and then modified by anti-CD20 adsorption. *In vitro* study: To investigate the ability of NP to kill Burkitt lymphoma cell lines, 5 x 105 BJAB cells were incubated for 48 hours and the number of residual viable cells was determined with MTT assay. Apoptosis was evaluated by analyzing PARP-1 cleavage and Annexin V detection. In vivo study: A xenograft model was developed in SCID mice inoculated i.p. with 2 x 106 labeled or unlabeled BJAB cells; a fraction of mice was treated with NP and all animals examined twice weekly up to 120 days. A small-animal time-domain eXplore Optix pre-clinical imager was used for the in-vivo and ex-vivo evaluation of labeled BJAB cell dissemination and Ritcy5.5 distribution. At 120 days the animals were sacrificed and peritoneal mass and organs, lymph nodes and bones were collected for immunohistochemical analysis. *Results. In vitro* study: The binding NP on cell surface and their internalization were documented with confocal microscopy. In BJAB cells, HCQ and CLM as free substances induced cell death in 82% and 38% respectively, while NP containing only 5,4 g of HCQ and CLB killed 100% of cells. This effect is due to the synergic pro-apoptotic effect as shown by Annexin V detection and by PARP activation. The effects of NP was tested on CD20-negative cell lines CHO, HUVEC, MEL-28, LoVo: No cell cytotoxicity was detectable using NP, whereas free HCQ+CLB induce cell killing between 58 and 71%. In vivo study: The intra-peritoneal injection of BJAB cells induced a fast (within 4 days) tumor cells spread in different organs and, without therapy, all animals died in 50-70 days as well as those receiving free HCQ and CLM. Conversely, survival at 120 days of the mice receiving 4 or 8 i.p. injections of NP was 50 and 90% respectively. *Conclusions.* These results indicate that NP are able to cure even the highly proliferating BL cell line BJAB in both *in vitro* and in vivo models. This suggests the introduction of this promising technology into human clinical trial.

CO-078

ANALYSIS OF THE CODING GENOME OF SPLENIC MARGINAL ZONE LYMPHOMA REVEALS MUTATIONAL ACTIVATION OF NOTCH2 AND OTHER PATHWAYS REGULATING MARGINAL ZONE DIFFERENTIATION

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Introduction. Splenic marginal zone lymphoma (SMZL) is a B-cell malignancy of unknown pathogenesis. This study aimed at characterizing the SMZL coding genome and identifying novel genetic alterations of this lymphoma. *Methods*. Tumor and germline DNAs from 8 discovery SMZL were subjected to whole exome sequencing (WES) (HiSeq 2000, Illumina). Genes identified through WES were investigated for mutation recurrence by Sanger resequencing in an independent panel of 109 SMZL. *Results*. In the 8 discovery SMZL, WES identified 203 somatic, non-silent mutations (mean, 25.3/case), and high density SNP array identified 41 somatic CNAs (mean, 5.1/case). When combining mutations and CNAs, the overall load of tumor-acquired lesions ranged from 13 to 60 lesions/case (mean, 30.5 lesions/case) (Figure 1A). Genes targeted in SMZL point to the involvement of programs implicated in normal marginal zone (MZ) development (NOTCH, NF-kB, B-cell trafficking) and chromatin remodeling (Figure 1B). Genes of the NOTCH pathway were recurrently mutated in ~32% (37/117) SMZL. Activating mutations of NOTCH2 represented the most frequent lesion in SMZL (25/117, 21.3%). Other NOTCH pathway genes mutated in SMZL were NOTCH1, a paralog of NOTCH2 (6/117, 5.1%), SPEN, a NOTCH repressor (6/117, 5.1%), and DTX1, a NOTCH modulator (2/117, 1.7%). Mutations of NF-kB regulators accounted for 40/117 (34.1%) SMZL, and targeted IKBKB (8/117, 6.8%), TNFAIP3 (8/117, 6.8%), CARD11 (8/117, 6.8%), BIRC3 (6/117, 5.1%), MYD88 (6/117, 5.1%), TRAF3 (4/117, 3.4%), and MAP3K14 (1/117, 0.9%). SWAP70, that is necessary for B-cell trafficking across spleen compartments, was disrupted in 4/117 (3.4%) SMZL. Mutations of genes implicated in MZ development show a largely mutually exclusive pattern and collectively account for the majority of SMZL (70/117, 59.8%), suggesting that they represent alternative mechanisms converging on the deregulation of a common downstream target (Figure 1C). A second set of genes recurrently mutated in SMZL regulate chromatin remodeling (14/40 cases, 35.0%). Overall, these mutations were frequently concurrent in the same patient, suggesting that they cooperate to promote tumorigenesis. Affected genes included MLL2, a histone methyltransferase (6/40, 15.0%), ARID1A, a member of the chromatin remodeling family (4/40, 10.0%), the acetyltransferase EP300 (2/40, 5%), SIN3A, a component of

the SIN3/HDAC1/2 histone deacetylase complex (3/40, 7.5%), and TBL1XR1 a component of the SMRT-N-CoR transcription corepressor machinery (4/40, 10.0%). *Conclusions*. Genetic alterations associated with SMZL pathogenesis predominantly involve signaling pathways that regulate MZ development. The results of this study provide a rationale for the design of novel therapeutic strategies for SMZL, since the NOTCH and NF-kB pathways represent two attractive candidates for target therapy with drugs already available or under clinical development for other diseases.

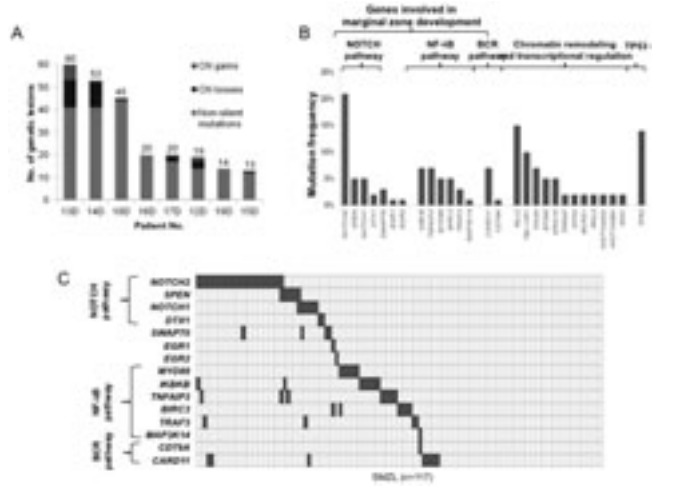

Figure 1.

CO-079

ADDITION OF RITUXIMAB TO INVOLVED-FIELD RADIOTHERAPY PROLONGS PROGRESSION FREE SURVIVAL IN STAGE I-II FOLLICULAR LYMPHOMAS: A MULTICENTRIC, RETROSPECTIVE SURVEY

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Radiotherapy (RT) is considered the standard treatment for stage I-II, non bulky, Follicular Lymphoma (FL). Despite initial therapy, most patients relapse especially outside the radiation field. To reduce the risk of disease recurrence, many authors have suggested to extend the irradiation field or to combine systemic chemotherapy to RT. However, both these approaches are associated with increased risk of early and late toxicity. Thus, the use of involved-field (IF) RT remain widely employed in stage I-II FL. The anti-CD20 monoclonal antibody rituximab (RIT) is extensively used in FL, with proven efficacy and excellent tolerability, either alone or in combination with chemotherapy. It should be effectively combined with RT, as well. Aims: to evaluate retrospectively the safety and efficacy of rituximab, administered in combination with RT as initial treatment for localized FL. *Methods.* from July 1999 to June 2011, 43 consecutive patients were treated for grade I-II FL, with stage I-II non-bulky at five Italian Hematologic centers. The treatment included 4 weekly infusions of rituximab at a dose of 375 mg/m2, followed by IF-RT. Main patient characteristics were: median age = 50 years (range: 34-82); M/F ratio=26/17; FLIPI 0/1 = 35/8; Bulky = 0; stage I / II = 37/6. The median dose of RT was 30.6 Gray (Gy) (range 20-40). Bone marrow PCR to detect molecular disease was performed in 32 patients: 10 (31%) were PCR positive. *Results.* the treatment was generally well tolerated, the addition of rituximab did not result in significant side effects except for some mild allergic reactions at the first infusion. All patients responded to therapy, with 42 (98%) complete remissions (CR) and 1 (2%) partial remission (PR). At a median follow up of 6.4 years (range: 0.4 to 12.2), the PFS was 69%, and overall survival (OS) 95%. As of December 2011, 41 of 43 patients (95%) were alive and 30 (70%) are in continuous CR; there were two deaths at 3 and 4 years for lung cancer in two smoker patients; 12 patients (30%) recurred (6 were PCR+ at diagnosis, 4 PCR- and 2 had no probe). These results were compared with a matched historical group of 46 patients with similar clinical characteristics and treated with IF-RT alone. The median PFS of RT alone vs RIT-RT was 5.2 years versus not yet reached. As shown in Figure 1, the 5-years PFS of RT alone vs RIT-RT was 52% and 69% respectively(P=0.032); the 5-years OS was similar 92% and 94%. *Conclusions.* Rituximab followed by RT-IF is a well tolerated treatment for patients with stage I-II FL; this approach offers prolonged PFS, higher than that observed in subjects treated with RT alone. The long-term results with RIT-RT are similar to those reported with the combination of RT to chemotherapy, but with significantly reduced side effects. The results suggest the usefulness of randomized phase III study designed to compare prospectively RIT-RT vs. RT alone in limited-stage FL.

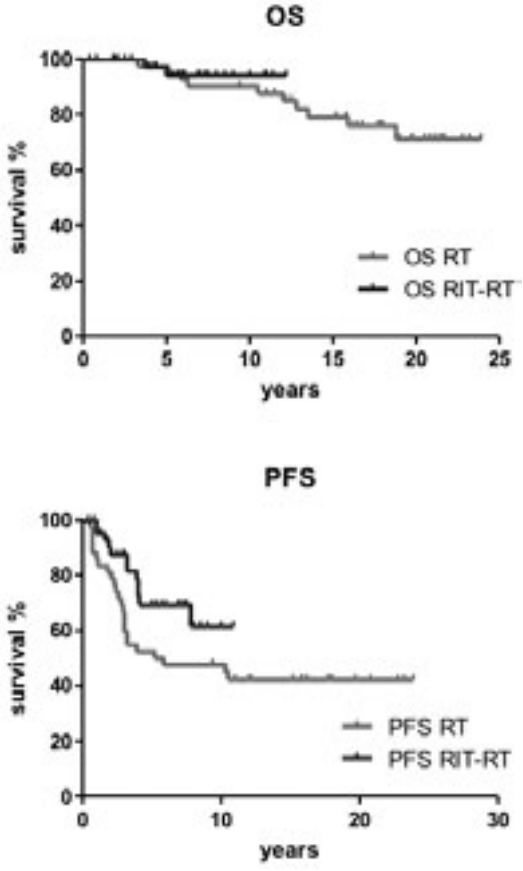

Figure 1. Overall survival (OS) and progression free survival (PFS) of patients treated with Rituximab and involved-field radiotherapy (RIT-RT) and patients receiving only radiotherapy (RT). OS P=NS; PFS P=0.032.

CO-080

HIGH ERP5/ADAM10 ESPRESSION IN LYMPH NODE MICROENVIRONMENT AND IMPAIRED NKG2D-LIGANDS RECOGNITION IN HODGKIN LYMPHOMAS

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Introduction. Stress-related immunity can be triggered by hematological malignancies, through the activation of the NKG2D receptor on CD8+T and gammadelta (gd) T lymphocytes, by NKG2D-ligands (NKG2D-L), as the MHC class-I related molecules MIC-A/B and the UL16-binding proteins 1-4 (ULBPs), expressed on cancer cells. However, NKG2D-L can be shed, interact with NKG2D on effector lymphocytes and hinder the recognition of tumor cells. Proteolytic cleavage of MIC-A has is known to depend on the thiol isomerase ERp5 and the disintegrins and metalloproteinases ADAM10 and ADAM17, which also cleave ULPBs. Overexpression of these enzymes has been reported in several tumors. In turn, soluble (s)NKG2D-L and cytokines produced at the tumor site, as the transforming growth factor (TGF)beta, down regulate the expression of NKG2D on effector lymphocytes, contributing to tumor escape. Moreover, plasma levels of sNKG2D-L correlate with disease progression in multiple myeloma, CLL, NHL and acute myeloid leukemias. *Methods*. Lymph node (LN) bioptic specimens were obtained from 25 patients with classical Hodgkin's lymphoma (cHL), provided informed consent and approval by the institutional ethical committee and compared to 15 tumor free sentinel LN. The following experimental procedures have been performed: i) co-culture of gd and CD8+ T cells with mesenchymal stromal cells (LNMSC) obtained from LN cell suspensions; ii) cytotoxicity against lymphoma cells, untreated or treated with drugs able to induce NKG2D-L (as sodium valproate, VPA); iii) gd and CD8+T cell TGFbeta production and secretion and sNKG2D-L release (by cytofluorimetric analysis and ELISA); iv) expression of ERP5, ADAM10, NKG2D-L in situ by histochemistry and Q-RT-PCR. *Results*. In cHL, the LN stroma displayed in situ high levels of transcription and expression of ERp5 and ADAM10, able to shed NKG2D-L from the cell membrane. These enzymes were detected both in LNMSC and in Reed-Sternberg (RS) cells; also, MIC-A and ULBP3 were present in culture supernatants of LNMSC or RS cells. NKG2D-L-negative RS cells could not be killed by CD8+T or gdT cells, unless RS cells were treated with VPA. Upon co-culture with LNMSC, CD8+T and gdT cells strongly reduced their cytolytic activity against NKG2D-L+ targets, due to TGF-beta, present at the tumor site, produced *in vitro* by LNMSC and able to down-regulate T lymphocyte expression of NKG2D. In addition, CD8+T and gdT cells from the lymph nodes of cHL patients co-cultured with LNMSC, underwent TGFbeta-mediated down regulation of NKG2D. *Conclusions*. In cHL the tumor microenvironment is prone to inhibit the development of an efficient anti-tumor response, through the inhibition of NKG2D-mediated activation of effector lymphocytes. Both RNA interference targeting TGFbeta and prevention of NKG2D-L release, including blocking of ERp5-binding domain on MIC-A, or inhibition of ERp5 or ADAM10 enzymatic activity, can be proposed as potential therapeutic targets.

Acute Leukemias 2

CO-081

DOES HTERT EXPRESSION LEVELS CORRELATE WITH KARYOTYPE COMPLEXITY IN AML?

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Telomerase is a ribonucleoprotein complex, that consists of two components, a telomere specific reverse transcriptase (hTERT) and a RNA component (hTERC). Telomerase deficiency is supposed to be associated with leukemia development, a paradoxical finding in view of the fact that leukaemia as most cancer is expected to over-express telomerase (Calado et al. 2009, Capraro *et al*. 2011). The goals of the present study were to estimate hTERT expression in a series of *de novo* AML patients and to correlate expression levels with chromosomal complexity, i.e. with normal versus complex karyotypes (defined by the presence of ≥3 defects). The ninety-six *de novo* AML patients were observed at our Institution within a three-years period. They were thirty-nine females and fifty-seven males; their median age was 59 years (range 18-84). On conventional cytogenetic studies fifty-one, whose median age was 57 years (range 18-78), presented a normal karyotype and forty-five, whose median age was 61 years (range 33-84), presented a complex karyotype. No significant difference in age between these two patients groups was observed (P=0.9). hTERT expression was determined in bone marrow samples by real-time reverse transcriptase polymerase chain reaction, using SYBR Green I. Primers' design was made using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). In order, to estimate hTERT expression levels in normal mononuclear cells, twenty-seven umbilical cord bloods (UCB) were examined (control group). Mean and median hTERT expression levels were 14.16 (standard deviation, sd=14.4, minimum value 0.4-maximum value 58.4) and 10.30 (Inter-quartile range: 4.5-17.3) respectively. In chromosomally normal AML mean and median hTERT expression levels were 7.98 (sd=14.35, minimum value 0.2-maximum value 71.2) and 2.85 (Inter-quartile range: 1.1-6.9) respectively whereas in chromosomally complex AML these values were 18.80 (sd=38.17, minimum value 0.2-maximum value 235.57) and 4.07 (Inter-quartile range: 1.8-20.4) respectively. When the Wilcoxon rank-sum (Mann-Whitney) test was applied to compare hTERT expression levels between UCB and chromosomally normal AML a significant difference was observed (P=0.001; 95% CI=-1.09-13.4) whereas when this same comparison was made between UCB and chromosomally complex AML no significant difference was observed (P=0.13; 95% CI=-20.2-10.9). In conclusion i) chromosomally normal AMLs present hTERT expression levels significantly different from those of UCB mononuclear cells; ii) chromosomally complex AMLs express hTERT at levels similar to those of UCB mononuclear cells. This finding is rather unexpected as genomic instability is supposed to be higher in complex than normal karyotypes. However, our data might strengthen recent observations suggesting an intriguing link between low hTERT expression and leukaemia development. Thus we are now evaluating whether this finding is produced by a loss-of-function mutation in the hTERT gene.

CO-082

MUTATIONS OF EPIGENETIC ENZYMES AND OF THE SPLICEOSOME MACHINERY ARE RARE IN THERAPY-RELATED LEUKEMIAS

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Introduction. Therapy-related leukemias (t-AL), including also t-MDS, are an increasingly recognized complication in patients previously treated with radiotherapy and/or chemotherapy for a cancer or autoimmune disease. t-AL display a high incidence of monosomal and complex karyotypes, p53 mutations, and gene-specific promoter methylation, but other AML-associated mutations, as FLT3, NPM1, CEBPA, and TET2 are usually rare. *Methods.* We studied the incidence of the recently described

mutations in "epigenetic" (IDH1 R132, IDH2 R140 and R172, DNMT3A R882), and in "splicing" enzymes (U2AF1, S34 and R35, SF3B1 exons 13-14 and 15-16, SRSF2 exon 1) in 101 patients with a t-AL (58 females and 53 males; median age 65 years, range 16-88). Given its role in DNA synthesis and methylation, we analyzed the prevalence of methylenetetrahydrofolate reductase (MTHFR) C677T and A1298C SNP, compared to healthy controls, matched for sex and age. MTHFR SNPs were studied using PCR-RFLP, while analysis of all other genes was performed on genomic DNA by Sanger sequencing (ABI PRISM 3100; Applied Biosystems). *Results.* We found 4 mutations in the IDH1 gene (4/95 pts, 4.2%, 3 R132C, 1 R132H), and 7 mutations in the IDH2 gene, (7/92 pts, 6.4%, 2 R172K and 5 R140) that were mutually exclusive. DNMT3A was mutated at R882 in 6 patients (6/94 pts, 6.4%, 4 R882H and 2 R882C, one of them was homozygous). The recurring K700 mutation in exons 15-16 of the SF3B1 gene was detected in 3 of 94 t-AL patients [K700E, 3.2%, none of them in t-AL secondary to a myeloproliferative neoplasm, (MPN)]. We did not find any mutation in SF3B1 (exons 13-14, n=65 pts) and U2AF1 (S34 and R35, n=94 pts) genes. Concurrent R140Q IDH2 and R882H DNMT3A mutations were observed in 2 patients. IDH1 and IDH2 mutations were significantly more frequent in t-AL secondary to a MPN (6/16 pts) when compared to t-AL with a previous solid tumor (5/47 pts), or with a previous lymphoproliferative disease (0/28 pts) (P=0.0011). DNA was available from the primary MPN in 5 pts. The IDH1/2 mutational status was identical between primary MPN and t-AL in 4 pts (1 mutated, 3 non-mutated), while 1 patient acquired the R172K IDH2 mutation. The pattern of mutations was similar in PB-mononuclear cells and CD34+ cells selected at the time of leukemic evolution in 7 patients. The homozygous 1298A>C MTHFR variant was more frequent in t-AL than in controls (7/49 pts vs 5/110 controls, P=0.03). In this line, haplotype frequency of the CC vs CA and TA vs CA was significantly higher in t-AL compared to controls ($p < 0.005$ for both), confirming a role for this enzyme in the susceptibility to secondary leukemogenesis. *Conclusions*. IDH1/2, DNMT3A, SF3B1 and U2AF1 mutations are rare in therapy-related leukemias, where mutations of other pathways and aberrant karyotype may play the dominant role. IDH mutations are frequent in t-AL secondary to MPN, confirming that disease evolution may be the pathogenic event in these forms.

CO-083

SCREENING OF TP53 (TUMOR PROTEIN 53) MUTATIONS IN B-ACUTE LYMPHOBLASTIC LEUKEMIA (B-ALL) REVEALS MISSENSE SUBSTITUTIONS, ABERRANT EXON-JUNCTIONS AND INTRON RETENTION EVENTS

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TP53 is a tumor suppressor protein that in humans is encoded by the TP53 gene. It responds to diverse cellular stresses to regulate target genes that induce cell cycle arrest, DNA repair, apoptosis, senescence or changes in metabolism. Tumour-associated alterations in TP53 are a hallmark of most human cancers and cause dramatic defects in p53 function. By contrast, only a small fraction, if any, of the >200 single nucleotide polymorphisms (SNPs) of TP53 in human populations are expected to cause measurable perturbation of p53 function. In humans, a common SNP involves the substitution of an Arginine for a Proline at codon position 72. Many studies have investigated a genetic link between this variation and cancer susceptibility, however, the results have been controversial. Since the pattern, frequency and significance of TP53 aberrations and SNPs in adult B-ALL have still to be determined, in this study we set-up a sensitive assay to address this issue. Mutations of TP53 were investigated in 69 adult B-ALL samples (60 Ph+ and 9 Ph-negative). Since the majority of the studies in leukemia were focused on DNA and resulted in low rate of TP53 mutations, we aimed to identify RNA mutations and aberrant isoforms. To this purpose three overlapping shorter amplicons covering the entire coding cDNA sequence (GenBank NM_000546.4) and the untranslated exon 1 [amplicon 1 (491 bp): exons 1-5; amplicon 2 (482 bp): exons 5-8; amplicon 3 (498 bp): exons 8-11)] and a longer amplicon (1,317 bp) starting from exon 1 and ending to exon 11 were sequenced by Sanger method. TP53 mutations were detected in 7 cases (10.1%), suggesting that these alterations are apparently rare events in B-ALL. They included 4 missense substitutions in the DNA binding and carboxyl-terminal tetramerization domains: C135Y (ex 5), A234T (ex 7), R290C (ex 8) and A347T (ex 10). Interestingly, in two cases aberrant transcripts were identified: 1) a TP53 isoform characterized by retention of introns 5-6-7 and predicted to encode for a truncated protein due a premature stop codon; 2) a TP53 isoform in which the DNA binding domain is lost due to an exon conjunction between the exon 4 and the 3' untraslated region (UTR)(ex4-3'UTR: 7579533-7572842, GRCh37/hg19). SNP P72R (rs1042522) was investigated in 77 adult B-ALL samples (68 Ph+ and 9 Ph-negative) and it was found heterozygous in 41.55 % (32/77) and homozygous in 54.55% (42/77) of cases. In conclusion, we demonstrate for the first time that TP53 alterations at the RNA level (missense substitutions, aberrant exon junctions and intron retentions) are highly frequent in adult B-ALL patients and that testing for TP53 mutations with a sensitive assay based on RNA analysis is required. Given the high frequency of rs1042522 in our cohort it will be intriguing to determine its role. *Supported by* ELN, AIL, AIRC, Fondazione Del Monte Bologna e Ravenna, FIRB 2006, Ateneo RFO grants, PIO program, PRIN 2008, Programma di Ricerca Regione–Università 2007–2009.

CO-084

VITAMIN D3 SENSITIZES NPM1-MUTATED AML CELLS TO ARA-C

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Introduction. AML carrying NPM1 mutations account for about one-third of adult AML, shows distinctive biological and clinical features and has been included as provisional entity in the 2008 WHO classification of myeloid neoplasms. It is generally characterized by high rate of complete remission upon standard induction chemotherapy and favorable prognosis when in absence of FLT3-ITD. Nevertheless, there are still patients that experience chemotherapy resistance, leukemia relapse and poor outcome, especially within the elderly patient population. Therefore new therapeutic strategies need to be explored. Cytosine arabinoside (Ara-C) is an antimetabolic agent, commonly used in the treatment of AML, which is incorporated into DNA when the cell cycle holds in the S phase and make cells unable to divide. Rapidly dividing cells are therefore most affected.

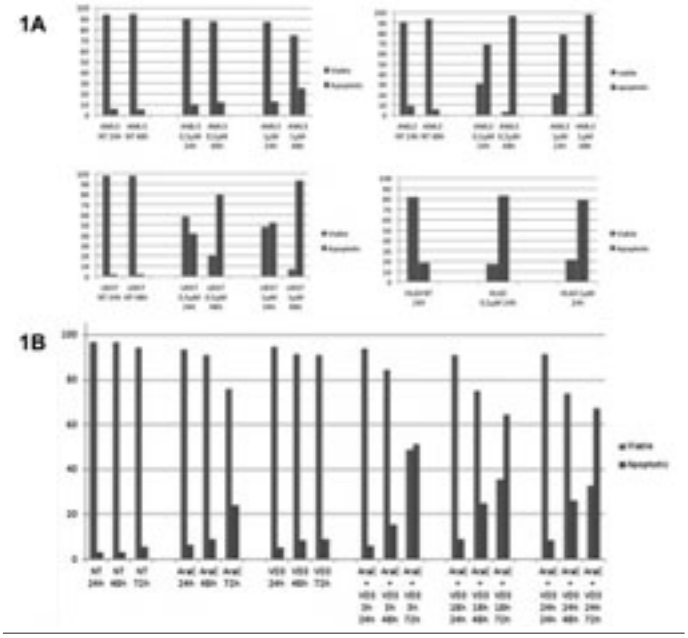

Figure 1.

Methods. Here, we studied the sensitivity of NPM1-mut AML cells to Ara-C and investigated how to potentiate its effects. As preclinical model, we used the OCI/AML3 cell line, previously identified as a human AML cell line carrying NPM1 mutation in the absence of FLT3-ITD. As

controls, we used the myeloid leukemia cell lines OCI/AML2, HL-60 and U937 not harboring NPM1 mutation. Cells were incubated either in absence or in presence of different concentrations of Ara-C (0.01 uM-1 uM) and effects on cell growth and survival were evaluated at different time points. In some experiments, cells were pre-treated with Vitamin D3 before adding Ara-C. *Results and Conclusions*. Surprisingly, although NPM1-mut AML have been reported to be generally chemosensitive, the OCI/AML3 cell line appeared less sensitive to the Ara-C induced pro-apoptotic effects when compared to the other human AML cell lines not harboring NPM1 mutation (Figure 1A). In fact, doses of 0.5-1 uM (pharmacological standard doses) appeared to be mostly ineffective. Increase of Ara-C doses (> 1 uM) lead to higher apoptosis rates, suggesting this might represent a strategy to ameliorate the outcome of NPM1-mutated AML patients. Interestingly, while not inducing apoptosis, 0.5 uM Ara-C blocked cell growth in OCI/AML3 and this was associated with upregulation of the cell cycle regulator p21. We hypothesized that the cell cycle arrest in OCI/AML3 might have a protective effect to Ara-C-induced apoptosis and investigated whether co-treatment with compounds known to promote cell cycle progression could sensitize OCI/AML3 to Ara-C. Interestingly, whilst Vitamin D3 itself had no effect on survival of OCI/AML3 cells, pre-treatment with Vitamin D3 (18-24 hrs) was able to markedly increase the apoptosis induced by Ara-C (Figure 1B). Preclinical experiments to confirm our findings on other NPM1-mutated AML cellular models and primary AML patient samples are ongoing. Our preliminary data indicate that drug sensitivity studies with 'conventional' and 'non conventional' drugs in different genetic subtypes of AML are warranted for a better tailored therapy in AML.

CO-085

RISK STRATIFICATION OF ACUTE MYELOID LEUKEMIA: A SICILIAN NETWORK FOR INTEGRATIVE ANALYSIS OF MULTIPLE MOLECULAR MARKERS AND KARYOTYPE

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Background. Acute myeloid leukaemia is a cytogenetically heterogeneous disorder with acquired recurrent chromosomal alterations detected in about 55% of patients. The remaining AML cases have normal karyotype (NK) and are characterized by an heterogeneous group of molecular abnormalities. In NK-AML molecular alterations with adverse prognostic impact include gene mutations of FLT3, WT1, IDH1, DNMT3A and high expression levels of the BAALC, ERG and MN1 genes, whereas favourable prognosis is associated with the presence of mutations in the CEBPA and NPM1 genes. *Aims.* We planned to perform a regional study involving all the Haematology Sicilian Centre with the objective of evaluating the incidence of the molecular aberration in our region and providing to the clinicians useful information about the biologic risk-category of patients. *Methods.* Cytogenetic studies were performed according to standard protocols. Molecular characterization included fusion gene (PML/RARalfa, BCR/ABL, AML1/ETO, CBFB/MYH11, MLL rearrangements); gene mutations (FLT3, NPM1, WT1, IDH1, IDH2, CEBPA and DNMT3A); gene expression (WT1, BAALC, ERG and MN1). AML1/eto and CBFB/MYH11 (CBF leukaemias) were also characterized for KIT gene mutation. *Results.* We enrolled 90 AML cases from across the region: 100 cases from western sicily and 90 cases from eastern sicily .We identified 13 APL carrying PML/RARAalfa fusion gene, the remaining 177 AML cases were so distributed: 38 AML NPM1 mutated (20%), 38 FLT3 mut (20%) (35 with FLT3 ITD and 1 with D835), 4 AML cases showed WT1 mutations, 9 AML cases showed R132C IDH1 mutations, 4 AML cases showed R172 IDH2 mutations, 8 CBF AML cases 1 of them showing D816V KIT mutation, no one AML showed BCR/ABL fusion gene, 1 AML case showed MLL rearrangements, 2 AML cases showed CEBPAA mutations, 12 AML cases showed R882H DNMT3A mutations. Cytogenetic studies available on 140 cases allow to identified 85 normal, 19 adverse, 15 intermediate and 21 favourable karyotype. Gene expression analysis of WT1 showed a median of 7304 copies/104 ABL copies (range from 2 to $26x10^6$). 50 NK-AML patients were divided into quartiles by gene expression levels of BAALC, ERG and MN1 and into low (I and II quartiles) and high expressers (III and IV quartiles). Patients were scored as high risk when showed high expression of almost two genes. Integrative analysis of a large molecular panel of markers and cytogenetic characteristics allow us to perform a prognostic stratification in high and low risk at disease onset in more than 80% of patients, only 10-20% of patients remained in the less informative "intermediate" category. *Conclusions.* Assessment of a exhaustive molecular and cytogenetic study at the presentation of the acute leukaemia may be very helpful to appropriately tailor the aggressiveness of therapy in the great majority of AML patients. This work was supported by a grant of Assessorato alla Salute Regione Sicilia (PSN 2011).

CO-086

MIR-155 REGULATIVE NETWORK IN FLT3 MUTATED ACUTE MYELOID LEUKEMIA

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Acute myeloid leukemia (AML) with normal kariotype (NK-AML) show, in about 30% of patients, FLT3 activating internal tandem duplication (FLT3-ITD), a mutation that confers unfavourable outcome. Our previous data demonstrated specific up-regulation of miR-155 in FLT3-ITD+ AML. miR-155 is known to be directly implicated in myeloid hyperplasia and/or hematopoiesis. Our aim was to integrate data from different source as GEO gene expression database and MIR@NT@N prediction tool to achieve a model about the role of miR-155 in FLT3-ITD+ AML and to validate this by experimental analysis. We applied a four steps strategy. At the first step, using gene expression dataset from GEO database, we generated the transcription factors co-regulation network acting in FLT3 mutated AML and at the same time, we predicted the miR-155-TF connections by MIR@NT@N. In the second step, we extracted, from the general network, the module of transcription factors connected to miR-155. At the third step, we compared the mir-155 module with the canonical pathways. At the final step, using cohort of newly diagnosed AML patients, we verified the expression levels of most intriguing hubs and correlated them to miR-155 expression levels. From these analyses, we derived a sub-network, called "miR-155 module" that describes functional relationship among miR-155 and transcription factor in FLT3-ITD+ AML. We found that "miR-155 module" is characterized by the presence of six transcription factors as central hubs: four miR-155 regulators (JUN, RUNX1, FOSb, JUNB) and two targets of miR-155 (PU.1, CEBPB) all known to be "master" genes of myelopoiesis. We found, in FLT3-ITD+ AML, a significant down-regulation of miR-155 target genes CEPB-beta and PU.1 (respectively 0.354 fold P=0.000 and 0.404 fold P= 0.000) and up-regulation of miR-155 regulator genes JUN and RUNX1 (2,597 fold P=0.0210 and 2,64 fold P<0.0001 respectively). We described, for the first time, a regulatory pathway that connects FLT3-ITD mutation to reduced expression of TFs master regulators of myelopoiesis. Our results suggest that activating mutation of FLT3 in AML can lead, through the induction of JUN, to increased expression of miR-155, which then causes down-regulation of PU.1 and CEBP-beta and consequently causes block of myeloid differentiation. In conclusion our study consolidates data on miR-155 association with FLT3-ITD+ AML, describes an integration of sequence-based prediction analysis with expression network that individuates vertices involved in the molecular pathogenesis of FLT3 mutated AML, suggests a molecular pathway that starting from in FLT3 activating mutation, through miR-155, damages myeloid differentiation. We also suggest that miR-155 deregulation may act as central hub in the multi-steps mechanism of FLT3 mutated leukemogenesis offering new therapeutic strategies. This work was supported by a grant of Associazione Italiana Ricerca sul Cancro (Project IG 10701 AIRC).

CO-087

MUCORALES-SPECIFIC T CELL MONITORING IN LEUKEMIC PATIENTS AT HIGH RISK FOR INVASIVE FUNGAL INFECTIONS

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Introduction. Invasive Mucormycosis (IM), the second-most common cause of invasive mold infections in hematologic patients, shows mortality rates approaching 70% of affected individuals, because of difficulties in obtaining an early and undoubted diagnosis. Actually, a definitive diagnosis of IM relies exclusively on both histopathological demonstration and cultural isolation of the pathogen from the involved organs. Adaptive immunity has been reported to play a crucial role in the host defence against fungi, at least in the case of invasive aspergillosis (IA) and invasive candidiasis. In particular, it has been shown that immune responses polarized to the production of IFNg may result protective to IA, while immune responses polarized to the production of IL10 may result non-protective, being permissive to IA. We have recently reported that the recognition and enumeration of Mucorales-specific T cells may serve as a surrogate diagnostic marker in patients with IM active disease (Potenza et al., Blood 2011). *Methods.* In the present study, we have used Elispot assay to assess the frequency of Mucorales specific T-cell responses producing IFNg, IL4 and IL10 in peripheral blood samples from 100 high risk hematologic patients. 74 out of 100 were patients receiving induction chemotherapy for acute leukemia and 26 out 100 were hematopoietic stem cell transplant patients. At least three samples for each patients were evaluated by Elispot assay using sonicated and heat killed germinated conidia from (Rhizopus Oryzae) Mucorales species, as antigenic stimulation. *Results.* 10 out of 100 (10%) patients showed the presence of a Mucorales-specific T cell response in, at least, one sample. Among these 10 positive patients, all the 5 patients without clinical features of Invasive fungal infection (IFI) showed specific IFNg-producing T cells, in association with lower levels of IL4 production in 2 of them. On the contrary, the 4 patients with possible IFI (according to EORTC criteria) showed positive results in more than one sample, with higher amounts of IL4-producing or IL10-producing T cells, in association with lower levels of IFNg production in 2 patients. Of note, the only patient with proven IM revealed higher amounts of IL4-producing Mucorales-specific T cells in three consecutive samples, in association with lower frequencies of IL10-producing and IFNg-producing T-cells. *Conclusions.* These findings suggest that Elispot assay could be a useful test to monitor the presence of Mucorales-specific T-cell responses in patients at high risk for IM. The detection of an immunological profile characterized by specific T-cell responses polarized to the production of type 2 cytokines, namely IL10 and IL4, could be the signature of active IM. Moreover, our data suggest that a T-cell response skewed to the protective IFNg production could be associated with a latent/controlled infection.

CO-088

LOW-DOSE LENALIDOMIDE IN ADDITION TO LOW-DOSE CYTARABINE INDUCES LONG-LASTING COMPLETE REMISSIONS IN A SUBSTANTIAL PROPORTION OF VERY ELDERLY ACUTE MYELOID LEUKEMIA PATIENTS

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We designed a phase II study to assess the antitumor efficacy of the combination regimen with low-dose lenalidomide and low-dose cytarabine in patients with acute myeloid leukemia (AML) aged more than 70 years. Thirty-three patients (median age 77 years, range: 71-85) were consecutively enrolled in the study. Median white blood cell count at diagnosis was $4.6x10^9$/L(range: 0.8-$46.8x10^9$/L), whereas median haemoglobin was 8.9 g/dL and median platelet count was $30x10^9$/L. Twelve out of 33 patients had a normal karyotype, 15/33 an intermediate or unfavourable karyotype and 2/33 were not evaluable. Thirteen patients had a *de novo* AML, whereas 20 patients had a secondary AML (14 after MDS, 3 after a CMPD, 1 after myelofibrosis, 2 after chemo-radiotherapy for a breast cancer). Patients received low-dose lenalidomide (10 mg/day orally, days 1-21) and low-dose cytarabine (20 mg twice day subcutaneously, days 1-15). Therapy was repeated every 6 weeks, up to 6 cycles. Six out of 33 patients died in aplasia while receiving the first induction cycle of therapy, and are not evaluable for response; one is not evaluable due to death while receiving chemotherapy (acute heart failure). Twenty-six patients completed at least one cycle of therapy and are evaluable for response. Among these patients, 11/26 (42%) obtained complete remission (CR) after the first cycle. Eight out of 11 responding patients are still in morphologic, cytogenetic and FISH CR after a median follow-up time of 13 months (range: 4-24 months). Two patients died while in CR after receiving, respectively, the second and the third cycle of therapy due to a multi organ failure after an infectious complication. One patient relapsed 3 months after the first cycle and died with active leukemia. The other 15 patients who completed at least one cycle of therapy did not respond at all and rapidly died due to progressive disease. At present, with a median follow up of 9 months for responding patients, 8/11 (72%) are alive in continuous CR, 2/11 died in CR and 1/11 died due to relapse. Notably, among responding patients, 7/11 (63%) presented with low WBC count at diagnosis (< 4000/mmc) and 9/11 (82%) had either an unfavorable (7/11) or intermediate (2/11; +8 abnormality) cytogenetics at diagnosis. In conclusion, low-dose lenalidomide has high activity in addition to low-dose cytarabine, in a subset of very elderly AML patients with extremely poor-prognosis. Considering the low compliance of very elderly, frail AML patients to high-dose therapy, this low dose schedule seems to be particularly profitable for patients with low WBC count and unfavorable/intermediate cytogenetics. The study was registered at EMA with the EUDRACT no 2008-006790-33. *Acknowledgments.* Celgene is acknowledged for providing Lenalidomide for the patients. The study was supported in part by AIL Pesaro Onlus.

Myeloproliferative Syndromes

CO-089

TARGETING HRASV12G EXPRESSION TO THE ZEBRAFISH EARLY HEMOGENIC PROGENITORS INDUCES A MYELOPROLIFERATIVE DISORDER BY REPRESSING THE NOTCH PATHWAY

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Introduction. Myeloproliferative diseases (MPDs) are a group of haematological disorders characterized by the hyper proliferation of different blood cells in peripheral blood and other hematopoietic organs. Given the genetic homology and the physiological similarity to mammals, zebrafish has emerged as an ideal model to study human normal and malignant haematopoiesis. In the last decade several Zebrafish models of hematopoietic neoplasms were developed with the aim to discovery new molecular pathways involved in malignant transformation. Despite the first encouraging results these experimental models failed to fully recapitulate human myeloproliferative disorders. *Methods.* We took advantage of the Gal4/UAS binary system to induce the expression of human oncogenic HRASV12G in the Zebrafish hematopoietic compartment. We used a specific transgenic line that drives oncogene expression in Zebrafish early hematopoietic progenitors under control of the FLI.1 (Friend Leukemia virus Integration 1) promoter. *Results.* We observed the development of a myelo-erythroid proliferative disease in few days in zebrafish transgenic larva. The pathological phenotype is characterized by the expansion of the hematopoietic tissue, an increase of expression of myelo-erythroid specific genes (PU.1, gata1, mpx, c-mpl) associated with a slight increase of staminality markers (lmo2, scl, c-myb, runx.1), and a higher number of l-plastin expressing cells. Moreover blood smear of pathological larva displayed leukemic blasts and the arrest of erythrocyte differentiation. We also found that the abnormal phenotype is associated with a down regulation of the Notch pathway as shown by the decrease of gene expression of notch pathways target genes (notch1, notch3, her6). *Conclusions.* The expansion of the zebrafish hematopoietic compartment characterized by the hyper-proliferation of the myelo-erythroid progenitors that we found in this model reproduces some of the pathological features of human myeloproliferative disorders. This study showed that forcing oncogene expression in the hemogenic compartment induces the trans-differentiation of the early hemogenic pluripotent stem cells into abnormal myeloerythoid progenitors by repressing the Notch pathways. In the future we will take advantage of this system to induce the expression of other human oncogenes known to be involved in hematopoietic malignancies.

CO-090

DUAL INHIBITOR OF PI3K/MTOR INHIBITS THE GROWTH OF CELLS OF MYELOPROLIFERATIVE NEOPLASMS (MPN) AND SYNERGIZE WITH JAK1/2 INHIBITOR RUXOLITINIB

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Introduction. Dysregulated JAK/STAT signaling, occurring mainly but not exclusively in cells harboring mutations in JAK2 or other proteins involved in JAK/STAT pathway, represents a pathogenetic event in MPN. Activation of other downstream pathways such as ERK and PI3K/mTOR pathway has been also documented in MPN cells. In this study we explored *in vitro* the potential relevance of targeting PI3K/mTOR pathway with a specific inhibitor BEZ235 alone or in combination with JAK1/2 inhibitor Ruxolitinib. *Methods.* BEZ235 and Ruxolitinib were provided by Novartis. The effects of drugs in cell lines were evaluated in liquid culture with WST1 assay. Quantification of apoptotic cells and cell cycle were done by flow cytometry. Protein expression levels were assessed by SDS-PAGE Western Blotting assay. CD34+ cells from MPN patients or control subjects were plated in semisolid medium. For single colony analysis, JAK2 genotyping was performed by ASO-PCR. *Results*. We found that JAK2V617F HEL and SET2 cells were sensitive to PI3K/mTOR inhibition (387±90 and 334±40 nM). We demonstrated that growth of JAK2V617F IL-3 or EPO-dependent (EPOR) Ba/F3 cells was inhibited at significantly lower concentrations of BEZ235 compared to WT counterpart (64±10 vs 10,000±500 and 87±50 vs 676±200). BEZ235 increased the fraction of G0/G1 cells at nM concentrations (<500 nM) but it was not effective in inducing apoptosis. Western blot analysis demonstrated that, as well as the inhibition of the mTOR target p4EBP1, BEZ235 also reduced the degree of pSTAT5 and pSTAT3. BEZ235 reduced clonogenic growth of MPN erythroid, myeloid and megakaryocytic progenitors at doses significantly lower (from 2 to 15-fold) than in normal cells, and prevented EPO-independent colony (EEC) formation (20±10 nM). Single colony genotyping in JAK2V617F patients showed a median of 48±16% (range 34-71%) reduction of JAK2V617F colonies in favor of WT colonies in the presence of 100nM BEZ235.Co-treatment of BEZ235 with Ruxolitinib resulted in synergistic inhibition of cell proliferation (Calcusyn software). Evidence of synergism was obtained in SET2 cells (CI: 0.37)and in Ba/F3 EpoR JAK2V617F-mutated cells (CI: 0.77). Activity of BEZ235 with JAK1/2 inhibitor was also assessed in a EEC assay. We found that addition of BEZ235 (3.4 and 0.2 nM) to very low dose of Ruxolitinib (0.02 nM) resulted in significant synergism (CI: 0.14). *Conclusion*. In summary, these *in vitro* data indicate that BEZ235 is active against MPN cells and its combination with Ruxolitinib resulted in synergism; studies in murine models are ongoing to confirm these results in vivo. Thus, concurrent targeting of PI3K/mTOR and JAK/STAT pathway might represent a new therapeutic strategy to optimize efficacy and reduce toxicity in patients with MPN.

CO-091

A DIFFERENTIAL CENTROSOME LOCALIZZATION OF WILD TYPE (WT) AND JAK2V617F PROTEIN IN HUMAN LEUKEMIA CELL LINES

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Introduction. The JAK2V617F mutation is frequently observed in myeloproliferative neoplasm (MPN). Vainchenker's group recently reported that the strong activation of JAK2-V617F stimulates homologous recombination, centrosome and ploidy abnormalities. The centrosome ensures symmetry and bipolarity of the cell division process, which is essential for accurate chromosome segregation and cell-cycle progression into mitosis. The centrosome amplification occurs frequently in both solid tumors and hematological malignancies and it is thought to contribute to the development of chromosomal abnormalities in these disorders. The level of centrosome tyrosine phosphorylation is a surrogate marker for activation of centrosomal downstream signaling pathways; constitutive phosphorylation at this site may perturbate centrosome function and cell cycle. *Methods.* To determine if JAK2 is a centrosomal partner, we performed co-immunoprecipitation and the co-immunofluorescence (CIF) assays between JAK2 and a centrosome marker g-tubulin on several cancer cell lines, derived from patient affected by CML (K562 and BV173, both expressing JAK2WT), or MPN (SET-2 and HEL cell lines, both carrying JAK2V617F). Moreover, we also evaluated JAK2 centrosomal localization in CD34+ cells isolated from MPN patients. Finally we evaluated if centrosome phosphorylation may be modulated by treatment with the JAK2 tyrosine kinase inhibitor AG490. *Results.* CIF assay shows a neat co-localization of JAK2 and -tubulin in K562 and BV173 cell lines as well as in CD34+ cells isolated from MPN patients with JAK2WT (in more the 90%±5% of cells) in a cell cycle independent manner. Moreover, we demonstrated that JAK2 centrosomal interaction is strictly dependent on the intact microtubule network, since nocodazole treatment inducing depolymerization of the microtubule network is able to reverse JAK2 centrosomal localization. By contrast, in leukemia cell lines carrying JAK2V617F in hemizygosis (SET) or in homozygosis (HEL), we detect colocalization of JAK2 and g-tubulin in only 40%±5% and 10±3% of the cells, respectively. In BM CD34+ cells isolated from MPN patients with JAK2V617F, we also observe a partial of JAK2 co-localization on centrosome. Notably, in 90% of HEL cells, lacking of JAK2-gTubulin co-localization, we identified high percentage (61%) structural and/or

numeric centrosome abnormalities. Moreover, the tyrosine phosphorilation of centrosome in SET and in HEL cell lines is abrogated by treatment with JAK2 inhibitor AG490. *Conclusions.* Our data suggest a functional interaction between JAK2 and centrosome in leukemia cell lines and in patients affected by MPN; this interaction may result in a genetic instability of neoplastic cells.

CO-092

A PRACTICAL APPROACH TO THERAPEUTIC DRUG MONITORING OF IMATINIB IN PATIENTS AFFECTED BY CHRONIC MYELOID LEUKEMIA. THE TIKLET PROTOCOL

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Introduction. Chronic myeloid leukemia (CML) has an incidence of 1-2 cases/100000/year, represents approximately 20% of all leukemias in adults, and it is characterized by the synthesis of the BCR-ABL1 protein. The introduction into clinical practice of drugs able to inhibit the tyrosine-kinase activity of BCR-ABL1 protein, as well as imatinib, had significantly improved the patients' survival. Recent studies have demonstrated that the highest therapeutic benefit is expected when minimal plasma concentrations (Cmin) of imatinib are equal or greater than 1 mg/L [Picard et al, Blood 2007; Takahashi et al, Clin Pharmacol Ther 2010]. However, imatinib pharmacokinetics is influenced by several physiological (age, body weight, plasma protein concentrations), pathological (liver and kidney function), and pharmacogenetic factors (polymorphisms of transmembrane transporters) [Petain et al, Clin Cancer Res 2008; Yoo *et al.*, J Clin Oncol 2010], while poor adherence to the schedule should be considered as an additional cause of treatment failure [Marin et al, J Clin Oncol 2010]. For these reasons, a therapeutic drug monitoring protocol should deserve particular attention in CML patients receiving imatinib. *Methods.* Fifty-two patients, 30 men and 20 women (mean±SD age, 56.5±11.8 and 54.2±14.2 years, respectively), affected by CML and treated with imatinib for at least 14 days were enrolled. Drug daily dose was 400 mg, except for three women (300 mg) and four men (600 mg). At different time points from drug administration, blood samples were obtained, centrifuged and plasma was analysed by a high performance liquid chromatography method with UV detection. The MWPharm software (MediWare, Holland) was used to obtain simulated imatinib plasma profile in each patient on the basis of measured drug plasma concentrations, according to a two-compartmental pharmacokinetic model. *Results*. Mean predicted peak and trough concentrations of imatinib were 2.44±0.60 and 0.80±0.44 mg/L, respectively. In particular, only 14 patients had Cmin values higher than the threshold of 1 mg/L. Pharmacokinetic analysis revealed a wide inter-patients variability for all of the parameters investigated. In particular, clearance (CL/F) and terminal half-life (t1/2) of imatinib accounted for 14.2±9.5 L/h (CV, 67.0%) and 16.1±5.7 h (CV, 35.1%), respectively, while volume of distribution (Vd/F) was 281.7±62.0 L (CV, 22.0%), and these results were in agreement with those previously reported [Widmer et al, Br J Clin Pharmacol, 2006]. Gender-related differences in pharmacokinetic parameters were not observed, except for Vd/F which was significantly greater in men (306.1±66.3 L) with respect to that calculated in women (248.4±35.3 L). *Conclusion.* The present results confirm that variability in imatinib pharmacokinetics is not negligible among individuals, and the adoption of a therapeutic monitoring protocol may help to identify those patients who could require a dose optimization.

CO-093

INTEGRATIVE ANALYSIS OF MRNA/MIRNA EXPRESSION PROFILES OF CD34+ CELLS IN PRIMARY MYELOFIBROSIS

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Introduction. Philadelphia-chromosome negative myeloproliferative neoplasms (MPNs) are a group of clonal hematological disorders that include polycythemia vera, essential thrombocytemia and primary myelofibrosis (PMF), which is the most morbid and frequently fatal MPN. Despite these diseases were clinically characterized more than 60 years ago, molecular mechanisms were partially unraveled only in 2005-2006 with the identification of somatic gain-of-function of JAK2 and MPL. Since then, many other mutated genes in MPN patients were found. Recently, several new molecular pathogenetic mechanisms were identified. Among them, aberrant microRNA (miRNA) expression especially seems to add up to the molecular complexity of MPNs, as specific miRNA signatures capable of discriminating MPN cells from those of normal donors were previously reported. *Methods*. In order to have a comprehensive picture of miRNA deregulation and its relationship with differential gene expression in PMF cells, we obtained mRNA and miRNA profiles in the same CD34+ cells from 31 healthy donors and 42 PMF patients by means of Affymetrix technology (HG-U219 and miRNA 2.0 arrays). Using a stem loop-primed reverse transcription system, we performed quantitative RT-PCR to validate selected differentially expressed miRNAs (DEM) found out by miRNA 2.0 arrays on a indipendent set of PMF and control samples. We performed an in silico integrative analysis with Ingenuity Pathway analysis software, which combines the computational predicted targets with the gene expression data, in order to construct regulatory networks of the functional human miRNA-target interactions. Results. We achieved a list of 74 human miRNAs modulated in PMF. Several miRNAs involved in hematological malignancies or known as oncomirs were upregulated (i.e. hsa-miR-155-5p, miRNAs belonging to the miR-17-92 cluster), whereas other aberrantly expressed miRNAs have never been described in the hematological context. The integrative analysis between differentially expressed mRNAs previously obtained and DEM disclosed a high number of predicted targets with anti-correlated expression to the trend of their targeting miRNAs. This approach allowed the identification of different networks potentially involved in PMF pathogenesis, such as disease progression, oxidative stress and chromatin remodeling. *Conclusions.* The integrative analysis allowed us to unveil the potential contribution of miRNAs through their targets to the pathogenesis of PMF. However, it will be necessary to understand whether DEM have a causal pathogenic role in PMF by means of overexpression and silencing experiments. Moreover, further studies will be required to validate the discovered miRNA-mRNA pairs, thus giving a functional interpretation in PMF disease context.

CO-094

PKC412 (MIDOSTAURIN) IS SAFE AND HIGHLY EFFECTIVE IN SYSTEMIC MASTOCYTOSIS PATIENTS: THE BOLOGNA EXPERIENCE

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Introduction. Mastocytosis is a myeloid neoplasm characterized by abnormal accumulation and frequent activation of mast cells (MCs) in various organs, expecially bone marrow, skin, liver and gastrointestinal tract. In most adult patients, the systemic form of mastocytosis (SM) is diagnosed, which includes an indolent, an aggressive and a leukemic subvariant. The c-kit mutation D816V is detectable in most adult patients with SM. Treatment of SM usually focuses on symptom relief by histamine receptor antagonists and other supportive therapy. However, in aggressive and leukemic variants, cytoreductive and targeted drugs must be applied. *Methods.* From 2008, 11 patients (male/female=3/8) affected by Mastocytosis, have been referred to our Institution. The median age was 53 years. All the patients underwent a bone marrow biopsy, with flow citometry and molecular biology analysis, in order to identify the presence of D816V mutation of c-Kit gene. Serum tryptase level was tested, resulting elevated in all cases. According to 2008 WHO diagnostic criteria, 9 patients presented with SM,

whereas in the other cases a skin isolated involvement was detected. Systemic symptoms were characterized by nausea, diarrhoea, asthenia, weight loss, pruritus and serotine fever, identifying an aggressive form of the disease in 5/11 patients, due to skeletal involvement in three cases, ascitis and liver function impairment in another one and bone marrow disfunction in the fifth one. Therefore, since a first line therapy with supportive care and histamine receptor antagonists wasn't followed by a significant benefit, a personalized use of PKC412 was asked and obtained for all these five patients. *Results.* From March 2011 three out of the five patients with aggressive SM have received a prolonged period of treatment with PKC412, which was administered orally, at the dosage of 100 mg twice daily, without rest periods. The drug was well tolerated. No serious adverse events were observed. All the patients obtained a quick and prolonged improvement of clinical symptoms, in terms of weight gain, bowel function and skeletal pain. At the bone marrow evaluation, the persistence of the D816V c-kit mutation was observed, despite a significant decrease of mast cell marrow involvement. In one case we observed, after a first good response, a disease progression, characterized by the sudden reoccurrence of the same symptoms detected at diagnosis, confirmed by a relevant expansion of a pathologic mast cell population in the bone marrow. After a rest period, the drug was readministered, and a second remission was obtained. *Conclusions.* PKC412 is safe and effective in patients with SM, being able to significantly improve not only the gastrointestinal and systemic symptoms, but also the haematological profile. The persistence of the D816V c-kit mutation, despite a morphologic remission, suggests that many other oncogenic factors may be responsible for the pathogenesis of the disease. *Acknowledgments*. Work supported by European LeukemiaNet, FIRB 2008, AIRC, AIL, COFIN, University of Bologna and BolognAIL.

CO-095

GENE EXPRESSION PROFILING OF CD34+ CELLS FROM PRIMARY MYELOFIBROSIS PATIENTS

Pennucci V,[1] Zini R,[1] Norfo R,[1] Salati S,[1] Bianchi E,[1] Guglielmelli P,[2] Bisognin A,[3] Rosti V,[4] Pietra D,[5] Ricci C,[6] Fanelli T,[2] Sacchi G,[1] Barosi G,[4] Cazzola M,[5] Rambaldi A,[6] Bortoluzzi S,[3] Ferrari S,[7] Tagliafico E,[7] Vannucchi AM,[2] Manfredini R,[1] on behalf of the AGIMM (AIRC-Gruppo Italiano Malattie Mieloproliferative) investigators

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Introduction. Philadelphia-negative chronic myeloproliferative neoplasms (MPN) enlist a spectrum of disorders that originate from deregulated clonal proliferation of hematopoietic stem cell and associated with overproduction of mature blood cells. These hematologic neoplasms comprise polycythemia vera, essential thrombocythemia and primary myelofibrosis (PMF). The molecular basis of MPNs has been recognized few years ago with the identification of somatic gain-of-function of JAK2 and MPL mutations. Only in November 2011 the U.S. FDA approved the JAK inhibitor ruxolitinib for treatment of intermediate and high risk myelofibrosis, including PMF. Despite that, the disease-initiating events remain elusive and our current understanding of the molecular pathogenesis of MPNs is still far from being completely defined. *Methods.* In order to characterize the molecular signature of CD34+ cells from PMF patients, we performed gene expression profiling (GEP) on 42 PMF samples, 15 bone marrow and 16 peripheral blood normal controls. GEP was carried out using the Affymetrix HG-U219 chip, expression raw data acquired using Affymetrix GeneChip Command Console Software and data processed with Partek Genomic Suite. *Results.* GEP identified a list of differentially expressed genes compared to controls. Many of the abnormally expressed genes are related to megakaryocyte (MK) differentiation, fibrosis and migration, all of these are processes involved in PMF progression. Of interest, we found the upregulation of some putative cancer markers, such as WT1 and ANGPT1, whose expression has already been associated with poor prognosis in hematological neoplasms and in other malignancies. Among the deregulated transcription factors, we detected several genes involved in the of CD34+ commitment, and potentially in their transformation, some of them already reported as deregulated in other hematological malignancies, such as FHL2 or KFL3. Biological effects of some selected genes, already validated by means of qRT-PCR in a independent PMF/control set, will be evaluated *in vitro* in normal CD34+ cells by overexpression/ silencing experiments. *Conclusions*. GEP and qRT-PCR have shown an aberrant expression of many genes possibly involved in some pathogenetic steps of PMF: genes related to MK development, chromatin remodeling, bone marrow fibrosis and oxydative stress. We have also observed deregulated expression of some trascription factors whose expression is correlated to hematopoietic malignancies. Furthermore, the altered expression of some putative cancer markers is particularly important for the purpose of our study, which aims at identifying potential PMF markers suitable as targets for new therapeutic strategies.

CO-096

JAK1 AND SOCS3 GENE EXPRESSION ARE PREDICTOR MARKERS OF RESPONSE TO INTERFERON-ALPHA (IFN) IN ESSENTIAL THROMBOCYTHEMIA (ET)

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Introduction. IFN is able to induce hematological response in about 70-80% of ET patients but some of them do not reduce plated count below $400x10^9$/L (and thus defined as bad responders). IFN- receptor lacks tyrosine-kinase activity and rely on Janus-kinase, Tyk2 and Jak1, for STATs phosphorylation and transmission of intracellular messages. The STATs activated in response to IFNs include STAT1, STAT2, STAT3 and STAT5. STATs proteins induce the transcription of SOCSs, whose role is to extinguish cytokine signaling by inhibition of JAK kinase-activity directly through the KIR-domain, and indirectly promoting the proteasomal degradation of Jak2, by SOCS-box-motif. IFN, inducing SOCSs expression, inhibits TPO mediated signaling through Jak2 inhibition. This study aimed to identify molecular markers to predict IFN treatment responsiveness in ET patients. We evaluated the mRNA expression of gene which signal cross-talks with the JAK-STAT pathway under IFN and TPO. *Methods.* We analyzed 54 ET patients treated with 3 million units of IFN--2b 5 times a week as induction (3 months), and 3 times a week as maintenance. Two groups of response were identified: Responders(R) (n=37), who achieved a reduction of platelet count below $400x10^9$/L, and No-Responders(NR) (n=17) who failed. For *in vitro* study, HEL and SET2 cell lines, carrying respectively homo and hemizygous JAK2V617F mutations, were treated with a dose escalation of IFN (1-10000 IU/mL). Proliferation rate and apoptosis were evaluated every 24 hrs for 7 days. The expression of JAK1, TYK2, STAT1, STAT3, SOCS1 and SOCS3 were explored in bone marrow samples of ET patients by RTq-PCR. Data were normalized as following: [mRNA normalized copy number (NCN)=mRNA target gene/mRNA GUSb*104]. *Results.* We detected JAK2 V617F mutation in 50% of R and 64,7% of NR (P=0,31) and no difference was found in JAK2V617F mRNA expression. Patients showed a median spleen volume of 500 ml in R and 300 ml in NR group (P=0.01). No other clinical characteristics were different between the two patient categories.Among all six investigated target mRNA, we detected a significant higher expression of JAK1 mRNA in NR than R (134465 vs 51405, respectively;P=0.000004). Also, NR showed a higher expression of SOCS3 mRNA than R (20141 vs 10585, respectively; P=0.0007). Thus, we confirmed our in vivo data also in an *in vitro* model of MPN. Indeed, SET2 cell line shows a significant inhibition of cell growth (P=0.03) and apoptosis induction (P=0.0002) upon IFN treatment than HEL cell line. JAK1 and SOCS3 mRNA expression was higher in HEL than in the IFN sensible SET2 cell line (P=0.009 and P=0.01, respectively). *Conclusions*. In vitro and in vivo data confirmed JAK1 gene expression as predictor marker of response to IFN in ET patients. Patients with low levels of JAK1 and SOCS3 mRNA may be eligible for IFN therapy. Moreover, patients with IFN-unresponsiveness molecular signature may be considered for JAK1/JAK2 inhibitor treatment.

POSTERS

Acute Leukemias 1

PO-001

SELECTIVE ANTI-LEUKEMIC ACTIVITY OF LOW DOSE DNA METHYLTRANSFERASE INHIBITORS AZACITIDINE AND DECITABINE ON AML1-ETO POSITIVE CELLS

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Introduction. Alterations in chromatin organization are a common mechanism in leukemogenesis. Many drugs affecting epigenetic modulation of gene expression have been proposed as therapy of hematopoietic neoplasms. We have demonstrated previously the selective action of histone deacetylases (HDAC) inhibitors on AML1-ETO positive cells(1). The AML1/ETO multiprotein complex induces and stabilizes the local DNA methylation pattern by recruiting HDACs and DNA methyl-transferase-1 (DNMT1), resulting in the stable silencing of AML1-controlled genes, like IL3 responsible of hematopoietic maturation(2). *Methods*. We analysed the effects of DNMT inhibitors (DNMTis), 5-Azacytidine (AZA) and Decitabine (DAC), at different doses (0.01, 0.1, 1 and 10 M) for 24h on AML1/ETO-inducible U937-A/E-9/14/18 cells, evaluating cell viability and apoptosis. We investigated in AML1/ETO-pos and neg U937 and HL60 (AML1-ETO negative) cells whether these effects could be specific and selective for AML1-ETO and thus correlated with silencing of IL3 gene. Cells were treated with AZA 1 M or DAC 0.1 M for 24h; we determined by ChIP assay the co-immunoprecitpitation of IL3 promoter with H4ac, H3K4me3, H3K9me2 and H3K27me3 and expression of IL3 by Q-RT PCR. *Results*. After AZA treatment the significative reduction of viable cells was observed at 0.1 M both in AML1-ETO neg and pos cells, but 0.1 M AZA significantly increased the percentage of apoptotic cells only in U937 AML1/ETO pos, confirmed also by the presence of cleaved caspase 9, 8 and 3. DAC 0.01 M reduced significantly cell viability in AML1/ETO negative and positive cells, but exposure to 0.01 M DAC significantly increased the percentage of apoptotic cells only in U937 AML1/ETO pos. After AZA and DAC treatment, we observed that increase of IL3 expression was higher in AML1/ETO-expressing than in non-expressing U937 cells. HL60 cells did not show any change in IL3 expression. By ChIP assay we showed that H3K4me3 (euchromatin) was not associated to IL-3 promoter, while H3K9me2 (etherochromatin) was associated to it, both in AML1/ETO-pos. and neg. U937 cells. H4-acetylated was linked to IL3 promoter only in U937 AML1/ETO-pos. cells after DAC treatment. H3K27me3 was linked to IL3 promoter in U937 AML1/ETO negative and in U937 AML1/ETO positive not-treated cells, but both AZA and DAC treatments lead to the dissociation of this modified histone from IL-3 promoter. *Conclusions*. These findings show that AML1-ETO positive cells are more sensitive to DNMTis than negative ones, regarding cell viability and apoptosis. Moreover, the dissociation of IL3 promoter from H3K27me3 (etherochromatin) and its link to H4ac (euchromatin) after DNMTis only in U937 AML1/ETO-pos. cells is indicative of the specific mechanism of action of these drugs on cells harbouring the chimeric protein.

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PO-002

FEASIBILITY OF A "CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA MOLECULAR DIAGNOSTIC PANEL" BY TARGETED NEXT GENERATION SEQUENCING.

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Introduction. In this study we have applied targeted Next Generation Sequencing (NGS) to evaluate the feasibility of a "childhood ALL molecular diagnostic panel" which could be able in a single procedure to detect point mutations, deletions and fusion genes known to have a role in childhood ALL. *Methods*. Genomic sequences of 43 genes with a known role in leukemogenesis were selected to cover approximately 5 Mb of genomic sequence. Genes involved in chromosomal translocations (ETV6, RUNX1, ABL1, BCR), mutations (JAK2, PAX5), and deletions (IKZF1, ADD3, EBF1) were included in microarray design. We sequenced the enriched genomic DNA of 2 childhood ALL cases: case n.1: t(9;22)/BCR-ABL1 positive BCP-ALL; case n.2: t(12;21)/ETV6-RUNX1 positive BCP-ALL. Briefly, 20 ug of genomic DNA at diagnosis was fragmented and hybridized onto the NimbleGen Sequence Capture 385K array (Roche NimbleGen, USA). The enriched sample was then sequenced by the 454 Titanium GS-FLX platform (Roche Applied Science). Raw reads from the 454 sequencing run were aligned against the DNA capture reference regions, by using Roche's own gsMapper tool. Fusion genes, indels, regions of loss of heterozygosity (LOH) and single nucleotide variations (SNV) were searched by applying custom bioinformatic pipelines. *Results*. For each sequenced case, the median gene coverage was 7.8 X and 11.5 X, respectively. Genomic sequences of BCR-ABL1 and ETV6-RUNX1 fusions breakpoints were identified in case 1 and 2, respectively. Reciprocal chimeric sequences were also found in case 1 between ABL1 and BCR genes, revealing a balanced translocation. In case 2, a homozygous deletion involving ADD3 gene was found. Moreover, the presence of a homozygous stretch of SNV in IKZF1 gene was found in case 1. SNP arrays analyses confirmed the homozygous deletion of ADD3 gene in case 2, and the presence of a hemizygous deletion of IKZF1 gene in case 1. An average of more than 7000 single nucleotide variations were found per case analyzed. Two exonic unknown variations were predicted to be translated in case 1. Only one of them resulted in a non-synonimous G/C variation affecting exon 10 of MLLT10 gene, predicted to result in a A292P aminoacidic substitution. *Conclusions*. We demonstrated that DNA sequence capture associated to NGS is able to simultaneously detect genomic breakpoints, gene deletions and single nucleotide polymorphisms/mutations of target genomic regions with high efficiency. We are aware that still the elevated costs per run, the complex and long time required for sequencing and interpreting the data represent limiting factors for routinely diagnostic application of this platform. However, the possibility to search, in a single procedure, for all genetic markers and aberrations of leukemic cells would represent a challenging goal to pursue, especially considering the expected cost decrease of NGS as well as the continuous development in data interpretation.

PO-003

MONITORING RESPONSE TO TREATMENT AND MINIMAL RESIDUAL DISEASE (MRD) BY FLOW CYTOMETRY IN ERDERLY PATIENTS WITH ACUTE MYELOID LEUKAEMIA TREATED WITH 5-AZACITIDINE: A SINGLE CENTER EXPERIENCE

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Introduction. Less than 20% of elderly patients are cured of acute myeloid leukemia with intensive chemotherapy.We analyze the efficacy and overall safety of 5-Azacitidine in elderly AML patients with severe co-morbidities ,correlated with immunophenotype data. *Methods*. From September 2009 to april 2012, 10 erderly patients (8 *de novo* and 2 secondary AML with median age 73 years, range 65-81 years) diagnosed with non-promyelocytic AML(not elegible for standard induction chemotherapy, signed informed consensus), received Azacitidine (75

mg/m²/d) for 7 days of every 28-day cycle until loss of response or disease progression in our institution. The patients presented severe co-morbidities (hepatopathy HCV correlated, ischemic cardiopathy, chronic renal failure, BPCO, diabetes), Cytogenetic analysis was performed and showed normal karyotype in 6 patients, in 3 patients monosomy of chromosome 5 and 7, in 1 patients chromosome 5q deletion. Immunophenotypic analysis of bone marrow samples was evaluated at diagnosis and during follow up (each six months) in 10 AML patients . Positivity was defined as more than 20% blasts expressing a specific antigen. It was possible to evaluate the percentage of positive blasts for the following antigens: CD33, CD13, HLA-DR , CD14, CD34, CD11b, CD117. *Results*. Median white blood cells was 4.4 x103 µL,range(1.2-16x10³ µL) The patients that during treatment down expressed positivity of surface antigen blasts <20% showed response of therapy. At oncet patients with acute myeloid leukemia had a medullary blast count of 20–50%.Patients received a median number of 14 cycles of therapy (range 7–24). For hematological improvement (HI) we considered stable disease but reduced need to supportive care(red cell and platelet transfusions).We evaluated overall response to treatment in 80%(CR 5pts + PR1pt + NR but HI 2pts),while 2 patients were non responder to treatment without haematological improvement. Generally the drug was very well tolerated. Adverse events in the overall study population were limited to grade 1 :neutropenia (60%) and thrombocytopenia (20%),gastrointestinal as constipation(10%), gastrointestinal hemmorage (10%). The median duration of response was 10 months with a range of 3-24months and overall survival with a range of 7-30 months with a median of 15 months. 2 patients died after relapse of disease after 24 months median duration response. Multiparametric flow cytometry (MPFC) is an alternative method to quantify MRD. The flow-cytometric abnormalities that characterize the leukemia blasts were reduced in PR patients and absent in CR patients. Besides using flow cytometry as monitoring method of MDR we noticed that in patients would lose the response to therapy after few months showed an increased flow cytometry positivity that predicted clinical outcome of patients (P<0.01). All patients with normal karyotype (P<0.001) reached complete and lasting remission. *Conclusions*. In our limited experience in these subgroups of erdely patients with AML, 5-azacitidine prolongs survival and is well tolerated even if used in patients with a higher degree of bone marrow blasts (>30%) and with severe co-morbidities in a subset of patients that could not tollerate AML like therapy but only supportive care .

PO-004

GEMTUZUMAB OZOGAMICIN (GO) AND AUTOLOGOUS PBSC TRANSPLANT: AN INTENTION TO TREAT ANALYSIS OF TWO POST-REMISSION STRATEGIES IN A COHORT OF ELDERLY AML PATIENTS.

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Elderly patients with AML has dismal prognosis, due to several factors, including high frequency of refractory disease and high relapse incidence. In order to reduce this latter we designed in this setting a prospective phase II study, based on two alternative post-remission strategies according to the PBSC harvest. Those AML patients collecting at least 3x10⁶ CD34⁺/kg received Autologous Transplant (ASCT) with Melphalan-including conditioning; poor mobilizer patients (CD34⁺ cells collection <3x10⁶/kg) received low-dose Gemtuzumab Ozogamicin (GO), 3 mg/sm monthly (three times) followed by 3 infusions every 3 months. Among 100 non-M3 fit AML patients, aged >59 yrs, who received an induction regimen, including Cytarabine and Idarubicine plus Amifostine, 72% achieved CR and 55 were eligible for PBSC collection. Patients' characteristics are shown in Table 1. A sufficient CD34⁺ cell harvest was achieved in 24/55 patients (44%). Seven patients were not eligible for post-consolidation treatment: 4 underwent Allogeneic Stem Cell Transplant from HLA-identical sibling and 3 received consolidation with intermediate-dose Cytarabine in pre-GO era. Therefore 48/55 patients in continuous CR (CCR) were assigned to the post-consolidation therapy with ASCT (N=23) or GO (N=25) on an intention to treat (ITT) basis. Twenty-one out of 23 patients received ASCT, (while 2 patients, relapsing before Transplant, died in early refractory relapse); 20 received ASCT in CCR and 1 in early relapse. After ASCT 5 patients died for early/late toxicity, 10 relapsed within 20 months from ASCT. Six patients are still alive and in CR with a median follow-up of 86 months and an 8 yrs OS and DFS of 25.4%. There was a significantly different outcome between patients mobilizing more and less than 7x10⁶/kg CD34⁺ cells in *high* and *normal* mobilizers (33.3% *vs* 21.4% 8 yrs OS and DFS). Twenty out of the 25 patients included in the GO ITT arm actually received it, five did not because of persistent aplasia, early relapse or poor PS. We did not observe any VOD, but only 5 transient liver enzymes elevation. Fifteen patients are still alive 12 in CCR with a median follow-up of 65 months and a 57% and 45.3% 8 yrs OS and DFS. Landmark Multivariate analysis identified post remission treatment and hyperleukocytosis as the only significant predictors of OS and DFS. In particular normal and high mobilizer patients, receiving ASCT, had respectively a 3.95 (95% CI: 1.62-9.63) and 1.6 (95%CI: 0.56-4.58) relative risk to die in comparison with patients receiving GO (P=0.01). Similar results were observed for DFS (Figure 1). *Conclusions*. Autologous Transplant feasibility and results are similar to those observed in literature, but GO, administered at lower dosage than those previously explored by the HOVON-SAKK phase III study, showed better results. Further phase III study should therefore evaluate a post consolidation role of GO at lower dosages and for longer administration.

Table 1. Patients' characteristics.

Patients characteristics	N (%)
Gender	
Male	58
Female	42
Karyotype	
Favorable	5(5.7)
Intermediate	49(56.3)
Unfavorable	33(38)
de novo AML	61
Secondary AML	39
Age	
<70 yrs	55
>69 yrs	45
WBC count	
<50,000/mcl	89
≥50,000/mcl	11
PS	
0-2	96
3	4
FDI	
0	60
>0	40
Sorror	
0-2	62 (74.7)
>2	21 (25.3)

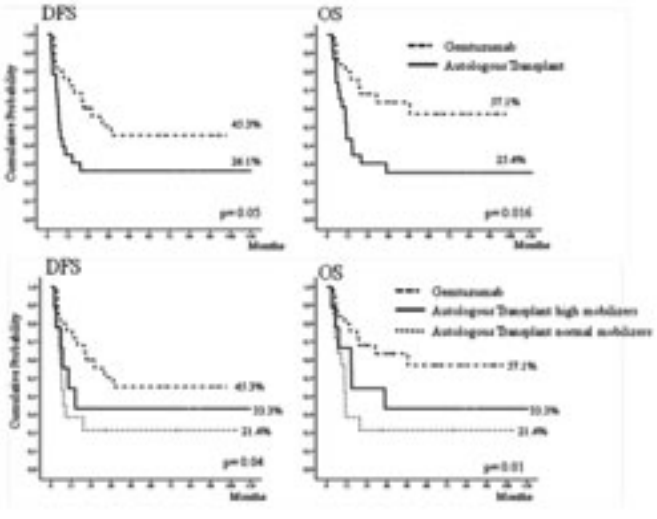

Figure 1. DFS and OS probability by post-remission treatment.

PO-005

MULTIPARAMETER FLOW CYTOMETRY (MFC) QUANTIFICATION IN MONITORING OF MINIMAL RESIDUAL DISEASE IN ACUTE MYELOID LEUKEMIA. PRELIMINARY RESULTS AT A SINGLE INSTITUTION

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Introduction. The evaluation of minimal residual disease (MRD) can be a reliable diagnostic tool to predict the prognosis in patients (pts) with AML and to choose post-remission treatment. In addition to PCR-based quantification of molecular abnormalities, multiparametric flow cytometry (MFC) has been proposed to study MRD through the identification of aberrant leukemia-associated immunophenotypes (LAIPs). MRD by MFC was studied and compared with molecular monitoring. Methods: From June 2010 to April 2012, in 37 *de novo*, unselected non-M3 AML pts (male/female 21/16; median age 49 years; range 18-73) with available karyotype (k), molecular (NPM1, AML1-ETO, CBFb-MYH11,CEBPA, FLT3 and WT1) and MRD analysis by MFC were evaluated. LAIP were identified on bone marrow (BM) samples at diagnosis, after induction and consolidation in pts treated according to GIMEMA-AML17 and NILG-AML00 protocols. According to MRC cytogenetic classification, 4/37 pts (10.8%) had favorable k, 26/37 (70.3%) intermediate k and 7/37 (18.9%) adverse k. By molecular evaluation, 12/37 pts (32.4%) were NPM1 positive, 9/37 (24.3%) FLT3-ITD positive and 3/37 (8.1%) CEBPA positive. WT1 was overexpressed in peripheral blood (PB) or in BM in 33/37 pts (89.1%) but WT1 levels allowed to detect a > 2-log reduction after induction therapy (Cilloni *et al.*, JCO 2009) in only 3/33 (9%). In 3/37 pts (8.1%) no specific molecular targets were identified and LAIP were not detectable in 4 pts concurrently seen, who were excluded from this study. For MFC detection of MRD an optimal cut-off value of 0.04% was defined, which was very similar to that reported (0.035%; Buccisano *et al.*, Blood 2010). *Results*. Complete remission (CR) was achieved in 74% (29/37 pts) after one (ICE or MICE) and in 100 % after two (SPLIT or FLAG) induction cycles. Median follow-up was 11 months (2-23). At CR achievement, 10/37 pts (27%) were MRD negative (<0.04%) and 27 (73%) MRD positive by MFC analysis. All MRD negative pts had achieved CR after the first induction. Nine of them (90%) had intermediate risk k, whereas 1/7 pts (14.3%) with poor risk k had undetectable MRD. Interestingly 4/4 CBF positive AML had detectable MRD both by MFC and by PCR. After consolidation, 30 pts were evaluable and 12 were MRD negative. Their relapse risk (RR) was 16.6% (2/12) compared to 38.8% (7/18) in MRD positive pts. Considering WT1 levels at CR achievement, the RR was 21.4% (6/28) in WT1 negative versus 25% (1/4) in WT1 positive pts. All pts were WT1 negative at the post consolidation. Their RR was 16%. At 20 months, survival was 83% and 65% and DFS was 67% and 54%, in MRD negative and MRD positive pts, respectively. *Conclusion*. Our preliminary data show that MRD value below the cut-off by MFC seems to correlate with the absence of unfavourable k and with CR achievement after a single induction course. Its prognostic impact on relapse risk needs to be analyzed on a larger number of pts and after a longer follow-up.

PO-006

USE OF CLOFARABINE IN PATIENTS WITH RELAPSED ACUTE MYELOID LEUKEMIA: A SINGLE CENTRE EXPERIENCE

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Introduction. Acute Myeloid Leukemia (AML) patients (pts) obtain complete remission (CR) in about 70% of cases, but relapse can occurre in most of them. Obtaining a second CR (CR-2) and sequential Bone Marrow Transplant (BMT) is the only approach to improve survival in these pts. Unfortunately there are not standardized chemotherapic regimens for relapsed AML. Clofarabine is a purine nucleoside analogue approved in second line for young pts with relapsed acute lymphoblastic leukemia. Experimental and clinical trials (CLASSIC 1 phase 2 study) suggest Clofarabine efficacy also in adult pts with relapsed AML.In this retrospective study we evaluated Clofarabine efficacy and tolerability in pts eligibled for a BMT. *Methods.* We analized 10 pts (3 females and 7 males)admitted in our department from September 2010 to September 2011. Median age was 46 years old (range 31-62), pts received Clofarabine (20 mg/mq) in combination with Cytarabine Arabinoside (1 g/mq) from day 1 to 5. All pts received antiviral and antifungal prophylaxis (Acyclovir 400 mg and Posaconazole 200 mg both thrice a day) but not any antibacterial prophylaxis. All pts suffered of a relapsed *de novo*-AML heterogeneously distributed in FAB classifications, 8/10 had a intermediate cytogenetic risk and 2/10 poor risk according to European LeukemiaNet criteria. All pts had a functional index ECOGPS 0-1 and 4/10 presented comorbidities (4 arterial hypertension, 1 with chronic hepatitis B, 1 dyslipidemia). *Results.* Overall response rate was 60% (4 CR and 2 partial remission). Overall survival after 1 year was 50%: 1 pt died in CR for hepatic failure; 2 pts died for persistent disease and 2 pts died for infective causes related to treatment before remission evaluation. 2/10 pts discontinued Clofarabine administration for hepatic toxicity (grade 3 and 4 - National Cancer Istitute v3.0): they received only 2 days drugs-administration and achieved CR. We noticed 14 non haematological adverse events: 8 sepsis (grade 3 NCI v3.0), 2 fatal septic shock during aplastic phase, 3 pneumonia and 1 invasive fungal infection. All pts experienced of a prolonged aplastic phase: median duration of severe neutropenia was 36 days (range 24-49). The 5 pts who survived and obtained CR-2 underwent to BMT, 3 of them received 2 courses of Clofarabine and Cytarabine and 2 only 1 course. *Conclusion*. Although our numbers are still small, we consider Clofarabine as a potential therapeutic option in pts with relapsed AML in order to obtain CR-2 (similar to the CLASSIC I study). After a median of 1.5 year of follow up 4 pts (40%) are still alive in CR after BMT. We found a high rate of life threatening infectious events (all our pts) probably due to prolonged and profound aplasia phase. We think that large comparative studies are needed to evaluate both Clofarabine efficacy and its side effects in relapsed AML pts.

PO-007

DIFFERENTATION OF LEUKEMIC BLASTS CORRELATES WITH THERAPEUTIC RESPONSE AFTER LOW DOSES OF RETINOIC ACID(LOATRA) +/- VALPROIC ACID(VPA) FOLLOWED BY LOW DOSES ARA-C (LODAC) IN ELDERLY (>65YRS)ACUTE MYELOID LEUKAEMIA:BIOLOGIC AND CLINICAL RESULTS OF A SINGLE CENTRE STUDY

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Introduction. Recent reports indicate clinical feasibility and biological potential of epigenetic treatments with VPA+ATRA or ATRA alone in AML, suggesting their capability in sensitizing leukemic cells to chemotherapy. Thus,we conducted a single centre study on the sequential administration of LoATRA +/-VPA and LoDAC (LoA-V-LoDAC)for the treatment of elderly or not eligible for intensive therapy AML patients(pts)The aims of this study were to asess clinical efficacy (in term of response rate and toxicity) and biological changes occurring in leukemic cells in relation to response.METHODS From September 2006 to November 2009, we enrolled 31 AML pts.(median age 72 yrs):17 *de novo*,5 secondary and 9 in ≥1 relapses.Twelve pts presented unfavourable molecular genetics(8complex kariotype,4FLT3+). Induction LoA-V-LoDAC schedule consisted of VPA(in escalating doses 10 mg/kg/die to 30mg/Kg/die)d1-55, LoATRA(LoA)25 mg/m d7-55; LoDAC 40 mg t.d./die s.c. d10-16 and 45-51,every 20 days up to 3 cycles.Twenty-one pts received LoA+LoDAC because of comorbidities,while only 10 were treated with LoA-V-LoDAC.PB and BM samples were sequentially collected at days 0, 7, 14, 28, 35, 55 to assess morphology, cytochemistry, immunophenotype,cell cycle and apoptosis, histone acetylation status, gene expression analysis. Response was evaluated after first cycle according to IWG criteria. *Results.* All pts were treated as out-patients.Of the 31 enrolled pts, 26 were evaluable for response,whereas 5 pts could not because they didn't reach at least 4 weeks of treatment.Hematological response was observed in 13/26 pts: 6 achieved CR, 6 PR and 1 mHI(ORR50%), 13 pts were non responders with 4 early deaths(ED)In the 13 responders, PB recovery and BM blasts clearance occurred at d42 (range35-50) and d35(range 25-50), respectively. Notably, response was

also observed in 5/12 pts with unfavourable molecular genetics. No pts presented grade 3/4 WHO extra-haematological toxicities. Biological studies revealed statistically significant differences in responders vs non responders:1) increased rate of cells in S phase and decrease in G1/Go phase (d+7 and d+14, P<0.05);2) cytochemical changes(increased MPO expression d+28, P<0.05);3) progressive decrease in early myeloid markers(d+28, P<0.05) expression and a concomitant increase of late myeloid differentiation markers (CD15 and CD64 at d+7,P<0.05; CD11b,CD16 or CD66 at d+14, P<0.05).Of the 13 responders,8 relapsed after a median of 7.5 m and 5 maintained PR for a median of 19 m (range14-40). As April 2012,median OS was 9 m (responders20.9,non-responders5,7m). To note, in the 5SD pts,OS was 16.2 m. *Conclusions.* Sequential LoA+/-V-LoDAC schedule proved to be a feasible, and safe approach to poor prognosis AML,and demonstrated the possibility to achieve therapeutic response even in pts with adverse molecular-genetics.Furthemore we provided evidences that LoA+/-V-LoDAC induced biological differentiation of leukemic cells significantly correlated with response.

PO-008

LOW-DOSE ARA-C COMBINED WITH ATRA IN ELDERLY PATIENTS AFFECTED WITH NPM1-MUTATED ACUTE MYELOID LEUKEMIA

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Introduction. In elderly patients with acute myeloid leukemia (AML), NPM1 mutations are associated with achievement of complete remission (CR) and seem to predict better outcome. It has previously been reported that elderly patients with NPM1-mutated (NPMc+) AML, without FLT3-ITD benefit from all-trans retinoic acid (ATRA) given in combination with conventional chemotherapy (Schlenk et al, Haematologica 2009;94:54-60). In older AML patients considered unfit for intensive chemotherapy, CR rates were significantly higher with low-dose Ara-C (LDAC) than hydroxyurea, but the benefit only applied to patients with intermediate risk cytogenetics. No beneficial effect from the addition of ATRA was observed, but it should be noted that no information about NPM1 mutational status was provided in that series (Burnett et al, Cancer 2007;109:1114-24). *Methods and Results.* We report here on two elderly patients affected with NPMc+ AML, without FLT3-ITD and with normal karyotype, admitted to our Department and considered unfit for intensive chemotherapy, because of advanced age and comorbidities. Patients received LDAC 20 mg twice daily by subcutaneous injection for 10 days. Subsequent course were administered after intervals of 4 to 6 weeks. ATRA 45 mg/m^2/day was administered for 60 days. Patient 1 is a 77 year-old man with a previous history of prostatic carcinoma and peripheral neuropathy, observed for pancytopenia. The bone marrow (BM) blast count was 40%. He was assigned to "good intermediate risk" prognostic group, according to Rollig et al (Blood 2010;116:971-78). The BM aspirate performed after 2 cycles of LDAC documented morphologic CR. After nine months from diagnosis, the patient have so far undergone 9 cycles of LDAC, combined with 3 ATRA courses, without complications. The complete blood count results normal and the BM aspirate still documents morphologic CR. Patient 2 was a 72 year-old woman with a previous history of arterial hypertension, hypothyroidism, depression and dementia. The BM blast count was >30%. She belonged to "good intermediate risk" prognostic group. The patient underwent 3 cycles of LDAC, combined with one ATRA course, obtaining full WBC and platelets counts recovery, concurrently with a reduction of RBC transfusion requirement. Unfortunately, on day +6 of the 4th LDAC cycle, disease progression was observed with WBC count 64.8x10^9/L and 70% circulating blasts, Hb 8.1 g/dl, Plt count 12x10^9/L). The patient died a few days later, 5 months from AML diagnosis. *Conclusions.* Experimental studies on OCI-AML3 and primary NPMc+ AML cells suggested that ATRA may induce cell cycle arrest and apoptosis by selectively down-regulating the mutant NPM1 protein. The combination of LDAC and ATRA may be effective in elderly patients, unfit for intensive chemotherapy, affected with NPMc+ AML, a relatively good prognosis AML, Prospective randomized studies are warranted to clarify the exact role of such treatment in this subgroup of patients.

PO-009

THERAPEUTIC MONITORING OF ITRACONAZOLE PLASMA LEVELS DURING ANTIFUNGAL PROPHYLAXIS IN PATIENTS WITH ACUTE LEUKAEMIA: RESULTS OF A PROSPECTIVE STUDY

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Introduction. Itraconazole is an azole widely used as antifungal prophylaxis in haematological patients (pts). Given the marked variability in the oral bioavailability and the possible interactions with concomitant therapies, we planned a pharmacokinetic study in haematological pts receiving prophylaxis with itraconazole, in order to evaluate the actual achievement of adequate plasmatic levels and the clinical efficacy in preventing invasive fungal infection (IFI). *Methods.* Consecutive pts affected by AML undergoing induction/re-induction or high-dose intensification chemotherapy, with a > 7 days expected neutropenia and receiving prophylaxis with itraconazole were enrolled. It was administered as oral solution or capsules (200 mg/bid). Plasma samples were collected weekly, before itraconazole administration, in order to perform therapeutic drug monitoring (TDM). *Results.* Twenty-eight AML pts were enrolled. Median age was 51.5 years (range 22-74). The total number of treatment cycles monitored was 34. Itraconazole was administered as oral solution in 19 pts (68%), as capsules in 9 (32%). Overall TDMs were 86. A concentration variability of itraconazole plasma levels between different pts and in the single pts was observed, particularly with oral solution, as a linear correlation between doses and plasma levels was not observed when capsule formulation was used. The steady state concentration of itraconazole plasma levels was reached after 14-21 days from the beginning of prophylaxis. The median concentration measured at the end of the 1st week (510±359 SD ng/mL) was significantly lower than that at the end of the 2nd week (690±494 ng/mL) (P=0.028), the 3rd week (1058±814 ng/mL) (P=0.0001) and the 4th week (1600±1442 ng/mL) (P=0.001). The percentage of pts with TDM below effective values (500±50 ng/mL) was 69.6% after 1st week, 40.7% after 2nd and 5.9% after 3rd. Despite the marked concentration variability, a correlation between the dose/kg/die and serum levels was observed, particularly for the oral solution formulation (R=0.712; p <0.001). Toxicity analysis during prophylaxis did not reveal a strict correlation between plasma levels and the onset of adverse events. However, we noticed a trend towards an higher gastro-enteric toxicity in the patients who reached the highest levels (1256 ±1163 vs 558 ±265). Four cases of IFI occurred during 34 cycles (11.8%), all observed during induction therapy, within the first 15 days from the beginning of prophylaxis. No direct correlation between incidence of IFI and plasma levels was observed. *Conclusions.* Our data confirm the potential usefulness of TDM of itraconazole plasma levels during prophylaxis. The delayed achievement of adequate plasma levels (after 14-21 days) from the start of prophylaxis together with the early onset of all cases of IFI observed concordantly suggest the future adoption of an initial loading-dose of oral itraconazole in order to optimize its cost effectiveness.

PO-010

CLINICAL AND MOLECULAR RESPONSE WITH AZACYTIDINE TREATMENT IN RELAPSED/REFRACTORY ACUTE MYELOID LEUKEMIA PATIENTS: REPORT OF A SINGLE CENTRE EXPERIENCE

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Introduction. Azacytidine is a demethylating drug approved for the treatment of high risk MDS and low bone marrow (BM) blasts count acute myeloid leukemia (AML). Several reports suggested its feasibility and efficacy also in AML patients (pts) unfit or relapsed/refractory. Here we report the clinical and molecular results of azacytidine treatment in relapse or refractory AML pts in our unit. *Methods*. From 2006 to 2011 we collected clinical data of 16 AML pts. Survival analysis was performed by Kaplan-Meyer method and response to treatment was evaluated according to Cheson criteria. Twenty-four BM samples from 16 pts were collected at baseline and during treatment; quantitative assessment of WT1 copies amount was performed according to ELN recommendations. *Results.* Pts' clinical characteristics at baseline were as follows:

median age 64 years (range 24-75), median BM blasts 13.5% (5-56), median WBC count 1940/mcl (680-5450), median neutrophil count 500/mcl (24-2850), median plt 76.000/mcl (18.000-258.000), median LDH 471 U/L (272-624). Eleven out of 16 pts had a relapsed disease and the remaining 5 were affected by a primary induction failure disease. The majority of them (69%) showed a standard cytogenetic risk, while the remaining pts were at high risk. WT1 values were increased in almost 69% of pts, median copy number at baseline was 665/104 abl copies (0-41441). Non one was eligible for further intensive regimens. Treatment schedule was 75 mg/mq for 7 days/months subcutaneously in an outpatient setting. Median number of cycles was 6 (1-15). Response to treatment was evaluated after 6 cycles of therapy or at progression. All pts were evaluated for final survival analysis. Infections were the most common extra hematological adverse events; among them 4 events were grade III-IV according to NCI CTCAE evaluation (3 pneumonia and 1 death for sepsis). CR, PR or HI was achieved in 6 out of 16 pts (38%). Half of them obtained a complete remission. Median duration of response was 4 months (0-13). Pts responsive to azacytidine shown a significant longer CR after prior regimens then non responders: 29 months (22-35) vs 10 (6-30), P=.04 by Mann-Whitney test. We failed to observe a statistically significant difference between responders and non responders pts considering baseline values of: BM blasts count, LDH, WBC and WT1 amounts. Complete molecular response was achieved in one patient with primary resistant AML and adverse cytogenetic risk after 6 cycles of treatment. Either median overall survival or median failure free survival were 14 months. *Conclusions*. In our experience azacytidine is generally well tolerated in relapsed or refractory AML pts and could be a feasible choice in subjects not eligible for salvage aggressive treatments. Duration of CR prior azacytidine is predictive of response. WT1 quantitative analysis is a useful tool for pts response assessment but not for pts' selection at baseline. Further data are needed to confirm our findings.

PO-011

COMPLETE REMISSION OF ACUTE LYMPHATIC LEUKEMIA , IN OLD PATIENT WITH COMORBIDITY, AFTER R - CVP PROTOCOL

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Introduction. Treatment recommendations for patients who are diagnosed with acute lymphoblastic leukemia (ALL) include induction, consolidation and maintenance therapy along with CNS prophylaxis. Newer studies with rituximab for CD20-positive ALL have resulted in 3-y survivals of 50% or more in adults. We report a case of LLA treated with R-CVP protocol that obtaining complete remission (RC) after 3 cycles. *Case report*. A 74 old year women , with heart's problem, was affected by ALL - preB without abnormalities of the karyotype (no Ph +) and positivity of blasts for monoclonal antibody CD20+. She was treated at first with cortisone in accordance with protocol for ALL and then with R - CVP modified/weekly, as induction therapy (Rituximab 375 mg / sqm on the weekly for 4 weeks) and subsequently every 21 days, obtaining the RC after 3 cycles in total. Therapy was well tolerated and was modified only the dose of the vincristine for neurotoxicity. Simultaneously meningeal prophylaxis with methotrexate and prednisone was performed. Following the revaluation, which documented the morphological and immunophenotypic complete remission has done a consolidation therapy according to the protocol L-VAMP for 3 cycles. No dose reduction of drugs for complications during consolidation. She used G-CSF during induction and consolidation therapy. During the intensive phase prophylactic anti-infective was introduced. The patient is going to revaluation of bone marrow: if RC will be maintained maintenance's therapy for ALL, with mercaptopurine, methotrexate and rituximab will be started. *Discussion.* CD-20 is an adverse prognostic factor in ALL. CD20 is a B-lineage antigen expressed on normal and malignant cells during nearly all stages of differentiation. It ranges from 40% to 50% in precursor B-lineage ALL compared with 80% to 90% in mature B-cell or Burkitt-type leukemia/lymphoma. The influence of CD20 expression on outcome for adults with *de novo* precursor B-lineage ALL was studied in the context of conventional [VAD] or intensive [hyper-CVAD] chemotherapy. Prospective randomized clinical trials incorporating rituximab into first-line therapy for precursor B-lineage ALL are planned. However, CD20 expression was associated with significantly higher relapse rates and lower 3-year CR duration and survival rates after hyper-CVAD therapy. *Conclusions*. The literature suggests that the expression of CD20 in adult ALL appears to be associated with poor prognosis but the incorporation of Rituximab in induction therapy may improve the clinical course of this patients. Our case is unusual because we obtained complete remission of LLA with Rituximab added to not conventional chemotherapy for LLA without anthracycline. This case could be an opportunity to use R-CVP protocol in very elderly and frail patients affected by acute leukemia with the same biological characteristics.

PO-012

REPROGRAMMING ACUTE LYMPHOBLASTIC LEUKEMIA CELL METABOLISM BY INHIBITION OF CARNITINE PALMITOYLTRANSFERASE 1A (CPT1A) PROTEIN

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Introduction. Tumor cells gain a survival and growth advantage by several mechanisms, including the metabolic change known as the "Warburg effect". In fact, by this profound biochemical alteration malignant cells preferentially use the up-regulated pathways involved in the aerobic glycolysis. It has been reported that Carnitine Palmitoyltransferase 1a (CPT1a), a protein that controls the fatty acid translocation across the mitochondrial membrane may play a fundamental role in this metabolic transformation. Aim of this study is to evaluate the expression of CPT1a in Acute Lymphoid Leukemia (ALL) cells, assessing the functional activity of CPT1a inhibitors, a developmental class of molecules with potential anti-tumor activity. *Methods.* Proliferation assay: MTT test. Protein expression: Western Blot (WB) analysis. Analysis of the cell-cycle and apoptosis levels: Acridine Orange technique and Annexin-V staining. *Results.* CPT1a expression, as determined by WB analysis, was measured in three T-ALL cell lines, the parental and the derived resistant CEM clones and the MOLT-4. CPT1a expression, detected in ALL cell lines, showed overexpression in clone CEM R. The functional activity of the novel CPT1a inhibitor ST1326 (kindly provided by Sigma-Tau) and of the well known inhibitor Etomoxir, was then *in vitro* evaluated. ST1236, at concentrations ranging between 1 and 50 M, induced apoptosis on the MOLT-4 cell line, in a dose and time dependent fashion, as demonstrated at 72 hours by an increase of the subG1 peak from a baseline value of 10% to 37% and 95.8%, at 20 and 50 M, respectively. A lower activity was found in the CEM S cell lines with an increase, at 72 hours, of the subG1 peak from a baseline value of 8.5% to 15.94% and 26.6%, at the same concentrations. Conversely, the CEM R cell line, proved resistant. These data were further confirmed by Annexin V staining. The effects on apoptosis induced by ST1326 were significantly higher ($p>0.05$) compared to those observed following Etomoxir exposure. Preliminary results on primary ALL blasts, obtained from 5 cases exposed *in vitro* to 10, 20 and 50 M of ST1326, showed an apoptosis induction (sub-G1 DNA content) from 38.9% ±23.7 (control) to 48.0% ±26.6, 53.5% ±30.6 and 68.9% ±23.4, respectively. The statistical significance ($P<0.005$) was however obtained only at the highest ST1326 concentration (50 µM). *Conclusions.* Overall, our results prompt the analysis of CPT1a expression in a large ALL sample population. In addition, the pro-apoptotic activity of ST1326 on ALL cells suggests that further studies aimed at validating CPT1a as a potential target for novel therapeutic approaches in ALL are needed.

PO-013

FLUDARABINE, CYTARABINE AND IDARUBICIN (FLAI) FOR INDUCTION TREATMENT OF POOR-RISK ACUTE MYELOID LEUKEMIA

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Backgroud. In an attempt to improve the outcome of AML, many strategies have been developed and tested. However, despite progressive intensification of the induction-consolidation treatment programs, no significant advantages have been observed in terms of CR rate, disease free-survival (DFS) or overall survival (OS) particularly in poor-risk patients. Poor-risk cases, that are identified mainly by prior history, leukemic cell mass and cytogenetic abnormalities, share multiple mechanisms of drug resistance that are responsible for treatment failure. *Methods.* In our institution we treated in the last two years 20 cases of poor-risk AML with fludarabine plus cytarabine and idarubicin regimen (FLAI). The FLAI regimen consisted of fludarabine 25 mg/m^2/d days1-5, Ara-C 2 g/m^2/d days 1-5, idarubicin 10 mg/m^2/d days 1, 3 and 5. *Results.* The complete remission (CR) rate was 85% after the first course and 95% after the second course. Non-hematologic toxicity was very mild, that is very important in elderly patients, but hemopoietic toxicity was substantial, with a time to hematologic recovery of 3 to 4 weeks and one case of death in CR. Peripheral blood stem cells (PBSC) could be mobilized and collected successfully in 18 cases. *Conclusions.* This three-drug combination is effective and has a limited non-hematologic toxicity, but fludarabine may increase the hematologic toxicity and enhance the development of serious opportunistic infections.

PO-014

NOVEL HISTONE DEACETILASE (HDAC) INHIBITORS: *IN VITRO* EFFECTS ON LEUKEMIC CELLS

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Introduction. Histone deacetylase inhibitors (HDAC-I) are a class of agents that have been intensively investigated in a variety of malignancies, because of their ability to inhibit proliferation, induce differentiation and apoptosis in tumor cells. However, clinical response have been obtained only in a proportion of patients, prompting further studies aimed at identifying more active compounds. Since acetyl-l-carnitine (ALCAR) is a metabolic intermediate that facilitates the influx/efflux of acetyl groups across the mitochondrial inner membrane, studies are ongoing to evaluate its activity as modulator of cellular stress response, in combination with chemotherapy. Here we investigated the effects of two HDAC-I, MS-275 and vorinostat (SAHA), alone or in combination with ALCAR, on cell proliferation and apoptosis in cell line models of haematological malignancies: acute myeloid leukemia (AML) acute lymphoblastic leukemia (ALL), and multiple myeloma (MM). *Methods.* HDAC-I were tested at doses ranging from 5 to 5000nM. In addition, the effects of simultaneous exposure to 10 mM of ALCAR and sub-toxic concentrations of HDAC-I were analyzed. The cytotoxic effect of the treatment was assessed by MTT assay. The drug concentration inducing 50% cell killing (IC-50) was calculated from the dose-response curve. Cell cycle inhibition and induction of apoptosis were analyzed by flow cytometry using the Acridine-Orange (AO) technique. *Results.* Comparative analysis of the efficacy of the two different HDAC-I indicated that MS-275 was the more effective agent with a clear dose-dependent activity, while SAHA displayed a flat dose-response curve, which dropped only at the highest concentration. In particular, the myeloid cell line Molm-13 was strikingly sensitive to MS-275 (IC50: < 15 nM), while the lymphoid cell line CEM R was resistant (IC50 > 10 uM). Other different cell lines exhibited intermediate sensitivity (IC50: < 1000 nM). SAHA showed no activity in U937 cells when used at concentrations ranging from 100 to 1000 nM, with a dramatic reduction of absorbance at 5000 nM (>80%). Nevertheless, the combination of 500 nM SAHA with 10mM ALCAR revealed a synergistic interaction, with a 46% reduction in absorbance. We then analyzed the effects on apoptosis induction. MS-275 dose-dependently induced apoptosis in HL-60 cells (4.2%, 17.1%, 60.8%, and 87.5% in the presence of 100, 500, 1000, 5000 nM MS-275, respectively). Conversely, SAHA induced minimal apoptosis (< 10%) at concentration ranging from 100 to 1000 nM, although > 75% of cells became apoptotic after treatment with the compound at 5000 nM. *Conclusions*. Our results show that the HDAC-I MS-275 is a potent inhibitor of leukemic cell growth, capable of inducing apoptosis particularly in AML and MM cell lines. Preliminary studies exploring the combined use of ALCAR with the SAHA support a potential anti-neoplastic synergism in hematological malignancies.

PO-015

LA DISREGOLAZIONE DEL GENE TRIB2 E' ASSOCIATA ALLA LEUCEMIA ACUTA MIELOIDE SCARSAMENTE DIFFERENZIATA DELL'ADULTO

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Background. Tribbles homolog gene 2 (TRIB2) is a pseudokinase gene belonging to a three member gene family. Tribbles gene was first identified in Drosophila melanogaster where is involved in the regulation of morphogenesis and mitosis. Likewise, mammalian homologous genes of Tribbles (TRIB1, TRIB2, TRIB3) promote the degradation of specific transcription factors and interact with several cell signaling mediators and modulators. Recently it has been demonstrated that TRIB2 was able to induce AML in bone marrow transplanted mice by inducing the proteasome-dependent degradation of C/EBP . Moreover, gene expression profiles data from AML patients revealed that patients carrying C/EBP mutations clustered with those patients with TRIB2 up-regulated. *Aims*. To test the hypothesis that the TRIB2 expression was associated to the differentiation degree in AML, we evaluated several cases based on FAB cytotype. *Patients and Methods.* We performed quantitative Real Time-PCR (qRT-PCR) analysis on bone marrow aspirate samples of AML patients (15 AML-M0, 14 AML-M2, 5 AML-M3 and 3 M5b) to measure the expression level of TRIB2. Healthy bone marrows were used as reference samples. The qRT-PCR was conduct using SYBR green chemistry, specific primers for TRIB2 transcript and two housekeeping genes (B2M and IPO8) previously tested. Each AML sample was tested by conventional cytogenetic analysis and by FISH using a TRIB2 specific probe. We performed, also, the mutational analysis of the TRIB2 coding sequence and the methylation analysis of a CpG island located in the TRIB2 promoter region through the methylation sensitive restriction enzymes (MSRE) and qPCR. *Results.* qRT-PCR experiments revealed that TRIB2 expression was higher (from 3 to 20 fold) in AML-M0 respect to the AML-M2 (P= 0.02), AML-M3 (P=0.01), AML-M5 (P=0.006) FAB subtypes, and references (P=0.003), respectively. Therefore qRT-PCR displayed a progressive decreasing TRIB2 expression across FAB subtypes. To disclose the reasons of such misregulation we performed further analysis. Conventional cytogenetic and FISH analysis did not show any kind of rearrangement involving TRIB2 gene as found in other cancers with TRIB2 up-regulated, and mutational analysis excluded the occurrence of activating mutations. MSRE experiments showed an higher methylation degree in a CpG island, located in the TRIB2 promoter region in AML-M0 cases when compared to the others FAB subtypes; moreover, methylation degree was significantly correlated to the TRIB2 expression (r=0.9; P=0.0002). *Conclusions*. Here we show that the TRIB2 expression correlate with the differentiation degree of leukemic cells in AML, and it is epigenetically regulated. In our cases the TRIB2 misregulation was not due neither to gene amplification nor to activating mutations. Our data reveal that TRIB2 gene promoter is differentially methylated according to the FAB subtypes. Usually, the methylation causes gene silencing but we have found that the methylation of TRIB2 promoter region was associated to the overexpression of the gene. Our hypothesis is that the methylation could inhibit binding of some unknown transcriptional repressor as already seen for hTERT gene in others tumors cells.

Chronic Myeloid Leukemia

PO-016

CO-EXPRESSION OF P190 AND P210 BCR-ABL FUSION TRANSCRIPTS AND RESPONSE TO TKI THERAPY IN CHRONIC MYELOID LEUKEMIA: THE RET (RETE EMATOLOGICA TOSCANA) EXPERIENCE

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Introduction. The hallmark of chronic myeloid leukemia (CML) is the presence of the Philadelphia chromosome and its resultant fusion gene BCR-ABL, and fusion protein, p210. Occasionally patients with CML can have a smaller BCR-ABL fusion transcript possessing only the first exon of BCR fused to ABL, resulting in a p190 protein, more frequent in Philadelphia positive acute lymphoblastic leukemia (ALL). A co-expression of both proteins, p190 and p210 has been described in CML, even in chronic phase; p190 mRNA usually is expressed at low level and may arise through alternative or missplicing. Its presence seemed to have no impact on prognosis in the pre-TKI era. *Methods.* We investigated the significance of co-expression of p210 and p190 fusion proteins in patients with CML in relation to disease features, therapy and outcome. Patients were monitored according to ELN recommendations. *Results.* 78 patients with new diagnosis of CML have been referred to our institution between 2008 and 2012, within the RET (Rete Ematologica Toscana) project. 8 cases (10%) expressed both transcripts. Median age was 53 years (range 31–66 years). At diagnosis 7 patients were in chronic phase (CP), 1 in accelerated phase (AP), none was in blastic phase (BP). Sokal risk was low in 2 patients, intermediate in 3 patients, and high in 2 patients. As frontline therapy, 5 patients received Imatinib 400 mg/day, 2 Nilotinib 600 mg/day and the one in AP Dasatinib 100 mg/day. The median follow-up was 16 months (range 3-47 months). Amount of p190 transcript was always low at the diagnosis (ratio BCR-ABL/ABL <1% in IS), becoming undetectable within 12 months of treatment in all patients; occasional, sporadic positivity at very low levels (ratio BCR-ABL/ABL ratio always in the range of MR4-MR.4.5 in IS) was frequently documented during the follow up , with no correlation with raise in p210 amount. Patient in AP obtained a CCyR (complete cytogenetic response) and a MMR (major molecular response) within 6 months of starting the treatment with Dasatinib; 2 of 5 patients in CP treated with Imatinib obtained a MMR at 12 months, a patient was resistant and switched to Nilotinib, a case at 12 months showed still a ratio of 1,468, while BCR-ABL/ABL ratio of the last patient with only three months of follow up was 12,198. Nilotinib as first line therapy induced a MMR at six months in a patient, while the other one has less than six months of follow up, but at 3 months he reached a CCyR, with a BCR-ABL/ABL ratio of 0,439. *Conclusions*. Co-expression of p210 and p190 transcripts is not a common event in CML, especially in chronic phase. Presence of low levels of p190 transcript at the diagnosis appears to be related to an increased Sokal risk and/or advanced phases, and to an apparent reduced probability of response to Imatinib. The majority of patients showed fluctuating, very low levels of p190 transcripts over the time, with no correlation with changes in p210 transcript amount.

PO-017

TREATMENT OF PH+ CML IN EARLY CHRONIC PHASE WITH SEQUENTIAL ADMINISTRATION OF NILOTINIB 400 MG TWICE DAILY AND IMATINIB 400 MG ONCE DAILY: A GIMEMA PHASE 2 MULTICENTRIC STUDY

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Introduction. The golden therapeutic standard for Ph+ chronic myeloid leukemia (CML) in early chronic phase (ECP) are tyrosine kinase inhibitors (TKIs) as single agents. Imatinib (IM) and Nilotinib (NIL) are TKIs approved as initial treatment of CML in many countries. A significant proportion of CML patients treated with single TKIs as monotherapy develops primary or secondary resistance. The use of more than one TKI may decrease the frequency of drug-resistance. *Methods.* To evaluate the response and the outcome of ECP Ph+ CML patients treated with the sequential administration of NIL and IM, a phase 2 study was conducted by the GIMEMA CML WP (ClinicalTrials.gov. NCT0076 9327). NIL was administered first because of faster therapeutic effect. Schedule: alternating administration every 3 months of NIL 400 mg twice daily and IM 400 mg daily; the 3-month rotation schedule was respected, irrespectively of temporary discontinuations. In case of toxicity, the patient remained in study, continuing the better tolerated drug alone. *Definitions*. Complete cytogenetic response (CCgR), absence of Ph+ metaphases over at least 20 metaphases examined by conventional banding analysis or <1% BCR-ABL+ nuclei over 200 nuclei examined by I-FISH; major molecular response (MMR), BCR-ABL <0,1%IS; failure, according to 2009 ELN criteria; event, failure or permanent discontinuation of both drug for any reason. All the calculations were performed according to the intention-to-treat principle. *Results.* 123 patients have been enrolled. Median age was 56 years; Sokal score: 33% low, 45% intermediate and 22% high; median follow-up was 27 months (minimum observation: 24 months). CCgR rates were: 72%, 79% and 75% at 3, 6 and 12 months, respectively; the cumulative CCgR rate by 12 months was 87%. The rates of MMR at 3, 6 and 12 months were 58%, 63% and 65%, respectively; the cumulative MMR rate by 12 months was 82%. The median time to CCgR and MMR was 3 months. At the last contact, 62% of patients were still on treatment with the alternating schedule, 9% were on NIL alone and 13% on IM alone. The overall survival was 94%, the survival free from progression to accelerated or blastic phase was 93%, the failure-free survival was 87% and the event-free survival 79%. The events leading to treatment discontinuation were: treatment failure 11%, adverse events 3%, death in chronic phase 2%, second neoplasia 1%, protocol violation 2%. Seven patients progressed to advanced phase: 3 cases with early transformation (within 6 months) developed BCR-ABL mutations (T315 in 2 patients and Y253 in the remaining patient), while 4 cases with later transformation do not. *Conclusions.* The response rates and short-term outcome are likely to be in the range of results achieved with TKIs as single agents, including 2nd generation drugs. A longer follow-up is required to assess the effect on the occurrence of BCR-ABL KD mutations. *Acknowledgements*. European LeukemiaNet, COFIN, Bologna University, BolognAIL.

PO-018

GLEEVEC-RESISTANT CML STEM AND PROGENITOR CELLS ARE SENSITIVE TO HIF-INHIBITING DRUGS

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Introduction. We previously demonstrated that incubation in severe hypoxia of blast-crisis Chronic Myeloid Leukaemia (CML) cells results in the complete suppression of BCR/Ablprotein, but not mRNA, so that hypoxia-selected Leukaemia Stem Cells (LSC) of CML, while remaining genotypically leukaemic, are phenotypically independent of BCR/Abl signaling and thereby refractory to the treatment with Imatinib-mesylate (IM). This is in keeping with the notion that IM is extremely effective in ensuring remission, but not cure. Cure indeed relies on the suppression of LSC, which most likely reside in hypoxic stem cell niches *in vivo* and therein sustain Minimal Residual Disease

(MRD). The main target of this study was to address the effects of the inhibition of Hypoxia-Inducible Factor 1 (HIF1) on the maintenance of hypoxia-selected LSC of CML. *Methods.* The BCR/Abl-independent maintenance of LSC in hypoxia has been assessed by the Culture-Repopulating Ability (CRA) assay. The maintenance of stem cell potential by CML cells selected in hypoxic primary cultures (LC1) is assessed by measuring their capacity to repopulate non-selective normoxic secondary liquid cultures (LC2). The CRA assay also allows to estimate the LC1 content with Leukaemia Progenitor Cells (LPC), by transferring to LC2 cells from LC1 at earlier times (days 2-3) than those necessary for LSC selection (day 7). The CRA of LC1 cells is estimated by determining the kinetics of LC2 repopulation. Two human stabilized CML cell lines were used, K562 and KCL-22. Three different drugs were used to inhibit HIF1: (a) a non-specific HIF inhibitor; (b) an inhibitor of coactivator binding to HIF complex; (c) an inhibitor of HIF1 dimerization with HIF1 . Cells were incubated in hypoxic LC1 (0,1 % O2) in the presence of one of the three drugs and transferred at different times into non-selective LC2. *Results.* Preliminary results obtained in our laboratory with K562 cells where HIF1 or HIF2 were suppressed by shRNA showed that BCR/Ablprotein-expressing LPC (rescued from day-2 LC1) were insensitive to HIF1 or HIF2 suppression, while BCR/Ablprotein-negative LPC (from day-3 LC1) were insensitive to the suppression of HIF2 but not HIF1 . The maintenance of hypoxia-selected LSC requires instead both HIF1 and HIF2. This indicates that the effects of shHIF are cell subset-specific. The treatment of hypoxic LC1 at time 0 or day 1 with chemical inhibitors (a) or (b) did not reduce the capacity of LPC or LSC to repopulate LC2. Inhibitor (a), however, but not inhibitor (b), resulted effective when administered for three days starting from day 6. Finally, inhibitor (c), given to LC1 at any time, completely suppressed LC2 repopulation by LPC as well as LSC. *Conclusions.* The detection of cell subset-specific effects of HIF1 inhibition led to propose some HIF1 inhibitors, together with IM, as a novel CML therapy targeting MRD via suppression of hypoxia-selected LSC. Data are being confirmed *in vivo*.

PO-019

HEAT SHOCK PROTEIN 90 (HSP90) REGULATES THE EXPRESSION OF PREFERENTIALLY EXPRESSED ANTIGEN OF MELANOMA (PRAME) IN CELL LINES DERIVED FROM PATIENTS WITH CHRONIC MYELOID LEUKEMIA

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Introduction. PRAME is a tumor antigen overexpressed in various malignant tumors including solid tumors and many hematologic malignancies, but is absent on normal tissues, including hematopoietic progenitor cells. In CML, PRAME expression was found to correlate with disease progression, since we demonstrated its increasing expression in blast crisis as compared to chronic phase CML patients. Moreover, several groups demonstrated that PRAME may significantly contribute to maintaining the tumor phenotype, since strongly associated with inhibition of cell differentiation induced by the all-trans retinoic acid (ATRA) receptor RAR-alpha, a crucial pathway for the proliferation and differentiation of both normal and malignant hematopoietic cells. Hsp90 is a molecular chaperone that plays a critical role in the folding, maturation and conformational stabilization of several oncogenic proteins. *Methods*. Several patients with CML in blast crisis and AML patients have high level of PRAME protein detected by Western Blot analysis, in spite of low PRAME mRNA expression, as assessed by Real-Time PCR. Thus, we speculate that PRAME protein might be protected from degradation by heat shock protein complex (Hsp90 and Hsp70). To evaluate if PRAME is an Hsp90 partner we performed the co-immunoprecipitation and co-immunofluorescence assays, on several leukemia cell lines, including K562 cell line derived from CML patient in blast crisis and expressing high level of PRAME protein. *Results.* We observed by confocal immunofluorescence microscopy a neat colocalization between PRAME and Hsp90/HSP70 proteins. Also we demonstrated that either Ab-PRAME co-immunoprecipates Hsp90 protein and Ab-Hsp90 co-immunoprecipites PRAME protein. Furthermore we also demonstrated that Hsp70 cooperates with the chaperone protein Hsp90 to bind PRAME. Concomitantly, we demonstrated that the expression of PRAME was significantly reduced in K562 cell line treated for 36h with 10μM of Hsp90 inhibitor 17-AAG [17-(allylamino)-17-demethoxygeldanamycin], confirming that the oncogenic protein PRAME is stabilized by Hsp90 binding. Moreover, the growth of leukemia cell lines *in vitro* was significantly reduced by 17-AAG exposition. *Conclusions.* Our preliminary data identify PRAME as a novel Hsp90 client and suggest that PRAME–Hsp90 interaction may have a crucial role to support leukemia cell survival. These findings may have significant implications for developing combinatory and effective therapies for leukemia patients and for offering a strategy to inhibit the oncogenic functions of PRAME by clinically available Hsp90 inhibitors.

PO-020

A RETROSPECTIVE REAL-LIFE ANALYSIS OF CHRONIC MYELOID LEUKEMIA PATIENTS IN SUBOPTIMAL RESPONSE TO IMATINIB: THREE CENTRES EXPERIENCE

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Introduction. Life expectancy of CML patients has greatly improved in tyrosine-kynase inhibitor (TKI) era, but still some questions remain about the management of suboptimal responders (SR) to imatinib standard dose. European Leukemia Net (ELN) recommended to continue imatinib in case of optimal response and to move to second generation TKI in case of failure but there isn't a clear agreement on SR: maintaining imatinib at standard or higher dose or switching to another TKI are all considered acceptable options (Baccarani et al, JCO 2009). *Methods and Results.* We retrospectively analysed 48 CML patients, diagnosed in chronic phase between 1988 and 2011, SR to imatinib 400 mg/d treated according to the 3 different ELN options. Forty-five patients received imatinib front line, 3 had been previously treated with interferon and cytarabine. Sokal score, evaluable in 33 patients, was high risk in 3, intermediate in 16 and low in 14, respectively. Eighteen patients were cytogenetic SR and 30 molecular SR according to 2009-ELN. The median follow-up from diagnosis was 79 months (range 10-292), only one patient died (gastric cancer). At suboptimal response detection 35 patients (72%) continued imatinib 400 mg/d, 11 increased imatinib dose to 600 mg/d (5) or 800 mg/d (6), 2 switched to new TKI (one was intolerant, the other obtained MMR). Among the patients who continued imatinib 400 mg/d (88% for at least 6 months), 8 (23%) major molecular responses (MMR) and 13 failures (37%) were detected. Thirteen patients maintained a suboptimal response and eventually increased imatinib dose (10/13) or changed TKI (3/13). Twenty-one SR patients increased imatinib dose, 60% at suboptimal response detection and 40% after further 12 months (median) of standard dose treatment (range 3-18). Fourteen patients (67%) obtained MMR, that was durable in 11 (52%). Four failures were observed (one after the achievement of MMR, one after intolerance to high dose imatinib). A total of 18 patients switched to a new TKI (13 dasatinib and 5 nilotinib), 12 after high dose imatinib, and 10 of 14 evaluables (71%) achieved MMR. At the end of the follow-up 36 patients (75%: 61% of the cytogenetic SR and 84% of the molecular SR) were in stable MMR; 7 more patients achieved complete cytogenetic response only. No patient progressed to accelerate/blastic phase. *Conclusions.* In our casistics no clear advantage in maintaining imatinib 400 mg/d after suboptimal response was observed (only 23% durable MMR, 37% failures). Imatinib dose increment led to a 52% of stable MMR and might represent a more reasonable option. The switch to 2nd generation TKI brought even better results than those obtained with high dose imatinib (70% stable MMR) in patients mostly unresponsive to the last option. Therefore, an earlier switch to new TKI might further increase the proportion of optimal responders. However, the small number of patients treated with new TKI in our study prevents definitive conclusions about the optimal management of SR.

PO-021

SAFETY AND EFFICACY OF BOSUTINIB IN CHRONIC PHASE CHRONIC MYELOID LEUKEMIA (CP CML)

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Introduction. Bosutinib (BOS) is an orally active, dual Src/Abl tyrosine kinase inhibitor in development for 1st, 2nd, and 3rd-line therapy of patients (pts) with CP CML. *Methods.* In the phase 3 BELA trial, adults with newly diagnosed (<6 mo) CP CML were randomized to BOS 500 mg/d (n=250) or imatinib (IM) 400 mg/d (n=252). In the phase 1/2 trial, adults with previously treated CP CML received BOS 500 mg/d after resistance/intolerance to IM only (CP2L cohort; <65 y, n=223; >65 y, n=63) or to IM plus dasatinib/nilotinib (CP3L cohort; <65 y, n=93; >65 y, n=26). *Results.* In the BELA trial of newly diagnosed pts, complete cytogenetic response (CCyR) was similar for BOS (56%) and IM (61%) at 30 mo; major molecular response (MMR) at 30 mo was also similar (45% vs 43%), although the cumulative MMR rate was higher for BOS (61% vs 52%). By 30 mo, 10% of BOS pts experienced an event-free survival event versus 14% of IM pts, including 3% and 6% of pts with on-treatment transformation to accelerated/blast phase (AP/BP) CML. Estimated overall survival (OS) rates at 30 mo were comparable for BOS (97%) and IM (95%). In the phase 1/2 trial, cumulative major cytogenetic response was high among both the CP2L (<65 y, 56%; >65 y, 43%) and CP3L (<65 y, 35%; >65 y, 27%) cohorts after median follow-up >30 mo; the majority of these pts achieved a CCyR. Estimated progression-free survival rates at 2 y were high in both the CP2L (<65 y, 83%; >65 y, 79%) and CP3L (<65 y, 76%;>65 y, 70%) cohorts; on-treatment transformation to AP/BP CML was reported for 3%-4% of pts across cohorts. OS rates at 2 y were also slightly higher for younger pts and those in the CP2L cohort. Gastrointestinal (GI) events (diarrhea, nausea, vomiting), were the most common toxicities with BOS; diarrhea was typically mild, occurred within the first month on therapy, and was transient (median duration of 1-3 days) across studies. Other common events included transient and manageable aminotransferase elevations and rash. In the BELA trial, BOS was associated with higher incidences of GI events and aminotransferase elevations and lower incidences of edema, musculoskeletal events, and neutropenia versus IM. *Conclusions.* Across trials, BOS demonstrated good clinical efficacy, with few on-treatment transformations to AP/BL CML and high estimated survival rates at 24-30 mo. BOS also demonstrated a manageable safety profile distinct from IM.

PO-022

SAFETY AND MANAGEMENT OF TOXICITIES IN THE BELA TRIAL OF BOSUTINIB VERSUS IMATINIB IN NEWLY DIAGNOSED CHRONIC PHASE CHRONIC MYELOID LEUKEMIA

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Introduction. Bosutinib (BOS) is an orally active, dual competitive Src/Abl kinase inhibitor. The phase 3 BELA study compared the safety and activity of BOS 500 mg/d with imatinib (IM) 400 mg/d in patients (pts) with newly diagnosed chronic phase chronic myeloid leukemia (CP CML). This analysis summarizes the safety profile of each agent, addressing management of gastrointestinal toxicities and liver function test changes. *Methods.* The median age was 48y (19-91 y) in the BOS arm and 47y (18-89 y) in the IM arm. Median treatment durations were 19.3mo for BOS and 19.5mo for IM; 67% and 74% of pts are still receiving therapy. The primary reason for BOS discontinuation was adverse events (23% BOS vs 6% IM). The primary reason for IM discontinuation was disease progression (4% BOS vs 13% IM). Deaths occurred in 2% BOS pts versus 5% IM pts; the majority occurred after treatment discontinuation. *Results.* BOS was associated with higher incidences versus IM of all grades of gastrointestinal toxicities and pyrexia. In contrast, BOS was associated with lower incidences of edema and musculoskeletal events. Diarrhea and vomiting were the most common grade 3/4 AEs. Elevation of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were reported as grade 3/4 AEs for 18% and 8% of BOS pts, respectively, versus 2% and 2% of IM pts. Diarrhea typically occurred during the initial month of treatment, with the median time to first event of 3d on BOS and 26d on IM and median duration of a diarrhea event of 3d and 5d respectively. Diarrhea was managed with antidiarrheal medication in 68% of BOS pts and 43% of IM pts; 22% and 9% of pts with diarrhea required temporary dose interruption, while 8% and 0% had a reduction of their dose. Of the pts who had a temporary dose interruption due to diarrhea, 34/38 BOS pts and 5/5 IM pts were successfully rechallenged. Grade 3/4 liver function test laboratory abnormalities were more common among pts receiving BOS versus IM, including elevation of ALT (23% vs 4%) and AST (12% vs 3%); the majority experienced grade 3 events. The median times to first ALT elevation were 28d for BOS and 114d for IM; median times to first AST elevation were 28d for BOS and 107d for IM. For BOS and IM, respectively, median durations for a grade 3/4 event to grade <1 severity were 21.0 versus 25.0d for ALT and 21.5 versus 25.0d for AST. Of the pts with ALT elevations, 35% versus 56% had a dose reduction and 56% versus 28% had a temporary dose interruption. 32/40 (80%) BOS pts were successfully rechallenged;4/4 IM pts were successfully rechallenged. Of the pts with AST elevations, 17% versus 5% had a dose reduction and 43% versus 16% had a temporary dose interruption. 26/26 BOS pts were successfully rechallenged; 3/3 IM pts were successfully rechallenged. 10 pts discontinued BOS due to ALT elevation. *Conclusions.* In conclusion, BOS and IM were associated with acceptable but distinct safety profiles in pts with newly diagnosed CP CML.

PO-023

EARLY EVALUATION OF THE RISK OF PROGRESSION TO ACCELERATED-BLASTIC PHASE IN CHRONIC MYELOID LEUKEMIA PATIENTS TREATED FRONTLINE WITH NILOTINIB

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Introduction. Nilotinib (NIL) is a second-generation tyrosine kinase inhibitor (TKI) that demonstrated higher and faster responses and lower progression rates compared to imatinib in early chronic phase chronic myeloid leukemia (ECP CML) patients (pts). Early cytogenetic and molecular responses have been associated to a better outcome for imatinib treat-

ed pts (Jabbour et al. Blood 2011; Marin et al. JCO 2011). Few data are available concerning the relationships between the rapidity of the responses and the probability of subsequent progression to accelerated-blastic phase (AP/BP) after NIL-based regimens frontline. *Methods.* The aim of the present analysis was to evaluate the rate of progression and to identify early predictors of progression in ECP CML pts treated with NIL-based regimens. Two hundred fifteen pts were enrolled in two multicenter phase 2 studies conducted by the GIMEMA CML WP (ClinicalTrials.gov. NCT00481052 and NCT00769327) or were treated at the Department of Hematology and Oncological Sciences of S.Orsola-Malpighi Hospital - University of Bologna, with NIL 400 mg BID or 300 mg BID as initial treatment. The median age was 53 years (range 18-86). The median follow-up was 29 months (range: 18-43 months). Definitions: complete cytogenetic response (CCgR) was defined as 0% Ph+ bone marrow metaphases by conventional banding analysis (≥20 metaphases examined) or <1% BCR-ABL nuclei in peripheral blood by FISH (≥200 nuclei examined, I-FISH); molecular response was defined as the BCR-ABL/ABL ratio according to IS; progression was defined as the transformation to AB/BP. All the calculations were performed according to the intention-to-treat principle. *Results.* Overall, 8/215 (3,7%) pts progressed, 7/8 during the first yea. At diagnosis 2 pts had clonal chromosome abnormalities; 2/8 pts had high EUTOS score, 6/8 intermediate (3) and high (3) Sokal score, 5/8 intermediate EURO score. At the time of progression the ABL mutational status was: 3 wild-type, 4 T315I, 1 Y253H. In order to identify early predictors of progression to AB/BP we analysed the cytogenetic and molecular response rates at 3 months in pts with and without subsequent progression. Seventy-four per cent (159/215) of the pts obtained a CCgR at 3 months, 3/159 progressed (1.9%); on the other hand, 26% (56/215) of the pts did not obtained a CCgR at 3 months (n=33) or were not evaluable (n=23), 5/56 (8.9%) progressed (P=0.0298)*. Eighty per cent (173/215) of the pts obtained a BCR-ABL/ABL ratio ≤1% at 3 months, 4/173 (2.3%) progressed; on the other hand, 20% (42/215) of the pts had a BCR-ABL/ABL ratio at 3 months >1% (n=23) or were not evaluable (n=19), 4/42 (9.5%) progressed (P=0.0487)*. *Conclusions.* In pts treated frontline with NIL-based regimens a CCgR and a BCR-ABL/ABL ratio ≤1% at 3 months correlated in univariate analysis with significantly reduced rates of progression to ABP. *Acknowledgements.* European LeukemiaNet, COFIN, Bologna University, and BolognAIL.

PO-024

SCREENING AND DEVELOPMENT OF NEW 14-3-3 INHIBITORS DISPLAYNG ACTIVITY IN PH+ CELL LINES

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Introduction. The standard treatment of CML is based on the use of tyrosine kinase inhibitors (TKIs) which target Bcr-Abl protein, the constitutively activated TK fusion protein encoded by the Philadelphia (Ph) chromosome. TKIs selectively bind to the ATP binding pocket of Bcr-Abl kinase, preventing its enzymatic activity and leading to tumor programmed cell death. The emerge of clinical resistance occurs in a minority of patients and nowadays is an ongoing challenge. 14-3-3 protein is directly involved in CML pathogenesis and in apoptosis, so it could be a new target for the development of novel and complementary therapeutic strategies to overcome drug resistance. In normal cells 14-3-3 forms a cytoplasmatic complex with c-Abl. In response to DNA damage c-Jun N-terminal kinase is activated and this causes the phosphorilation of 14-3-3 leading to c-Abl release. In this way c-Abl shuttles into the nucleus and promotes the apoptosis. In CML cells the presence of Bcr-Abl interferes with the dissociation of the 14-3-3 /c-Abl complex. So 14-3-3 inhibitors could enable the c-Abl cytoplasmatic release from the complex, its nuclear translocation and consequently its apoptotic effect. On the basis of the 14-3-3 inhibitors previously synthesized (BV01 and BV02), other computational calculations let us to select 15 new compounds as potential 14-3-3 /c-Abl protein-protein interactions inhibitors. *Methods.* To identify the most active compound in term of cytotoxic potential, preliminary cell proliferation assays (MTS) were performed in Ph+ K562 cell line. Compounds were evaluated both alone and in combination with TKIs to verify the putative synergic effects. The cytotoxicity driven by induction of apoptosis was measured by cytofluorimetric analysis by annexin V. *Results.* Some of the 15 compounds showed a decrease of cell viability in a range of 10-50% after 96 h with daily treatment. The rate of cell viability reduction was 25% using GV2-18 or GV2-85 compounds, 15% using GV2-84 or GV3-23 compounds and 10% for GV3-36 and GV3-9 compounds. The most active compound was GV2-20 which showed a decrease of cell viability of about 30% after 72 h and 50% after 96 h with daily drug additions. GV2-20 compound activity seemed to be higher than BV01, the most potent 14-3-3 inhibitor as previously reported. The preliminary cytofluorimetric analysis indicated that 20-40% of cells were apoptotic after treatment. *Conclusions.* This study let us to identify 7 new 14-3-3 inhibitors which interfere with cell viability and induce apoptosis in CML cell lines. We are performing protein analysis to verify the ability of different compounds in releasing c-Abl from 14-3-3 by promoting its relocation into the nucleus.

PO-025

DNA HYPERMETHYLATION PROMOTES THE LOW EXPRESSION OF PRO-APOPTOTIC BIM ASSOCIATED WITH BCR-ABL FUSION GENE OF CHRONIC MYELOID LEUKEMIA

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Introduction. Polymorphisms and/or downmodulation of genes involved in the response to tyrosine kinase inhibitors (TKIs) strongly influence the clinical response. One such candidate gene is BIM (Bcl2-interacting mediator otherwise named BCL2L11), which encodes a pro-apoptotic BH3-only death activator, a critical component of cell fate upon cytokine withdrawal. BIM induction is a key component of chronic myeloid leukemia (CML) progenitor apoptosis in response to imatinib (IM) (Kuroda et al, PNAS 2006;103:14907). Accordingly, its loss due to epigenetic events or deletion polymorphism protects leukemic cells from IM-induced death hence contributing to intrinsic drug resistance (San José-Eneriz et al, Eur J Cancer 2009; Ng KP et al Nat Med 2012: epub ahead of print). We have recently proved that the Bcr-Abl-expressing cell response to IM encompasses the 5 methylcytosine (5mC) conversion into 5 hydroxymethylcytosine at the BIM promoter (5hmC) (Mancini M et al, J Cell Biochem 2012: epub ahead of print). Notably, methylation of the 5th carbon of cytosines upstream of transcriptional start sites is a stable chemical modification associated with transcriptional repression, while 5mC hydroxylation promotes active de-methylation by impairing the recruitment of DNA methyltransferase (DNMT) 1 (Boultwood J et al, Br J Haematol 2007). Here we show that the recruitment of DNA methyltransferase (DNMT) 1 contributes with the loss-of-function of Ten-Eleven-Translocation (TET) 2 dioxygenase to BIM transcriptional repression associated with Bcr-Abl. *Methods.* We performed PCR amplifications of Chromatin Immuno-Precipitation (ChIP) products obtained with anti-DNMT1, -5mC and -5hmC antibodies at BIM promoter. We evaluated also the levels of BIM mRNA and protein in a 32D cell line stably transduced with Bcr-Abl and mononuclear cell fractions (MCFs) from bone marrow samples of CML patients compared to parental cell line and MCFs from healthy persons. *Results.* ChIP products obtained with an anti-DNMT1 antibody revealed DNMT1 increment at a 342 bp sequence of the BIM promoter (-268 to +90 region) associated with an excess of 5mC over 5hmC in a 32D cell line stably transduced with Bcr-Abl and mononuclear cell fractions (MCFs) from bone marrow samples of CML patients compared to parental cell line and MCFs from healthy persons. DNMT1 dislodgment from the BIM promoter associated with 5mC reduction and 5hmC increment was apparent since 2nd up to 6th h of *in vitro* exposure to IM and paralleled the Bcr-Abl protein de-phosphorylation at Tyr245, supporting that the gene hyper-methylation is contingent upon the Bcr-Abl protein tyrosin kinase (TK). It resulted in a progressive increase of BIM transcription followed by the increment of all three BIM isoforms and apoptosis induction. *Conclusions.* Our results confirm the CML methylator phenotype and support the combination of TK inhibitors and de-methylating agents, including decitabine or azacitidine, to improve the disease prognosis.

PO-026

ADDITIONAL CHROMOSOMAL ABNORMALITIES IN PH POSITIVE CLONE: ADVERSE PROGNOSTIC INFLUENCE ON FRONTLINE IMATINIB THERAPY. A GIMEMA WP ON CML ANALYSIS

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Introduction. Additional chromosomal abnormalities in Ph+ cells (ACAs) have been reported in about 5% of patients with newly diagnosed chronic myeloid leukemia (CML) in chronic phase (CP). Few studies addressing the prognostic significance of baseline ACAs in patients treated with imatinib frontline have been published previously. The European LeukemiaNet recommendations suggest that the presence of ACAs at diagnosis is a "warning" for patients in early CP, but there is not much information about their outcome after therapy with tyrosine kinase inhibitors. *Methods.* To investigate the role of ACAs in early CP CML patients treated with imatinib mesylate, we performed an analysis in a large series of 559 patients enrolled in 3 prospective trials of the GIMEMA Working Party on CML. *Results.* 378 patients were evaluable and ACAs occurred in 21 patients (5.6%): 5 cases with major routes abnormalities (trisomies 8 and 19), 9 cases with loss of chromosome Y, 6 cases with single different abnormalities and the last one with 2 ACAs. The cytogenetic and molecular response rates were uniformly lower in patients with ACAs: no significant differences were observed at 12th month, but the overall CCgR and MMolR rates were significantly lower in patients with ACAs: 71% vs 89%, P=0.03 and 67% vs 86%, P=0.03, respectively. The responses were significantly slower in the group of patients with ACAs. The median time to CCgR was 7 and 6 months in patients with and without ACAs, respectively (P=0.045). The median time to MMolR was 13 and 8 months in patients with and without ACAs, respectively (P=0.006). The estimated overall probabilities of EFS, FFS, PFS and OS were 52% vs 68% (P=0.096), 61% vs 76% (P=0.062), 85% vs 89% (P=0.453), 89% vs. 92% (P=0.764) for patients with and without clonal ACAs, respectively. *Conclusions*. In conclusion, this large series of patients suggests that ACAs at diagnosis, although not frequently detected, have negative impact in early CP CML patients treated with imatinib as frontline therapy. Our data confirm that patients with ACAs constitute a "warning" category, in term of responses, and suggest that these cases could require a close monitoring and treatment with second generation TKIs as front-line therapy. In agreement with a recent observation,[1] more intensive therapy should be considered in particular in patients showing major route abnormalities. *Aknowledgements.* Supported by Associazione Italiana contro le Leucemie, i linfomi e i mielomi Bologna (BolognaAIL); by Progetti di Ricerca di Interesse Nazionale (PRIN2007) University of Bologna; by the Fondazione del Monte di Bologna e Ravenna; and by the European LeukemiaNet.

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PO-027

THE LOSS OF NUCLEAR BETA CATENIN FOLLOWING CHIBBY ENFORCED EXPRESSION EVOKES ENDOPLASMIC RETICULUM STRESS IN CELLS EXPRESSING THE BCR-ABL FUSION GENE.

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Introduction. Chibby (Cby) is a -catenin antagonist encoded by the C22orf2 gene of chromosome 22 q13.1 (Takemaru K et al. Nature 2003;42:905). C22orf2 relative proximity to the Bcr breakpoint on chromosome 22 and beta catenin key role in Bcr-Abl leukemogenesis and self-renewal of leukemic stem cells (LSC) suggest Cby participation in the proliferative advantage of BCR-ABL+ hematopoiesis (Hu Y et al. Leukemia 2009;23:109). Indeed, we proved that C22orf2 located at the chromosome 22 involved in reciprocal translocation follows the Bcr downstream sequences and relocates to derivative chromosome or to the second fusion gene in variant tranlocations in Bcr-Abl+ leukemias. C22orf2 relocation was associated with Cby downmodulation most likely contingent upon reduced protein stability (Mancini et al, submitted). Here we report that beta catenin nuclear export and inactivation driven by Cby enforced expression evokes endoplasmic reticulum (ER) stress, a critical component of protein folding fidelity and activation of signal transduction pathways eventually leading to apoptotic cell death (Walter P et al. Science 2011;334:1081). *Materials and Methods.* We used K562 cell line stably transduced with a C22orf2 construct containing the whole gene coding sequence into a commercial plasmid (pcDNA3.1) encompassing the gene for neomycin (G418) resistance. C22orf2-transduced cells were evaluated for protein and transcript expression, proliferation and cell cycle distribution and ER stress induction after 8 week selection in medium additioned with 500 microg G418. *Results.* Higher levels of C22orf2 transcript and Cby protein were associated with a massive nuclear export of Beta catenin bound to the 14-3-3 scaffolding protein. Beta catenin transcriptional inactivation driven by its sub-cellular relocation resulted in a very significant reduction of proliferation rate (with doubling time upraised from 22.3 to 96.7 hrs) mostly contingent upon the induction of G2/M checkpoint. Moreover, it evoked the ER stress which is initiated by the combined activation of inositol-requiring enzyme (IRE1), release of protein kinase RNA (PRK)-like ER kinase (PERK) and increase of C/EBP-homologous protein (CHOP, otherwise named GADD153). The last protein participates in ER stress-corrective actions through either induction or suppression of downstream genes, such as Bcl-2, Bim and Bax, which may integrate other components of ER stress-mediated apoptosis in response to tyrosine kinase inhibitors. *Conclusions.* Our work lets assume beta catenin participation in life-or-death decision of hematopoietic progenitors and, more importantly, of leukemic stem cells expressing the Bcr-Abl tyrosine kinase

Reference

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PO-028

IN VITRO AND *IN VIVO* ANALYSIS OF T CELL PROLIFERATION AND ACTIVATION BY TYROSIN KINASE INHIBITORS (TKI)

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Introduction. TKIs are the current standard treatment in chronic myeloid leukemia (CML). In addition to the BCR-ABL target oncoprotein, they also inhibit off-target kinases (e.g. c-KIT, TEC, SRC), some of which have physiological functions in immune responses. Although *in vitro* studies have implied immunosuppressive effects of TKI treatment, comprehensive *in vivo* data are missing. Despite the myriad biochemical pathways that ultimately account for T-cell activation, the TCR proximal signaling pathway is essential given its primacy in the overall activation process. In particular, two Src family kinases (SFK), Lck and Fyn,

are involved in the very earliest steps of TCR activation, and Lck is suggested to be more important for TCR signaling. Thus, it is conceivable that agents targeting SFK activity could become useful adjuvants in therapeutic immunomodulatory regimens targeting aberrant T-cell activation. Indeed, Imatinib treatment has been used to treat sclerodermatous chronic GVHD (cGVHD), since inhibiting transforming growth factor b (TGFb) and PDGFb pathways in fibroblasts. *Methods.* Thus, we evaluate the activity of clinical relevant dose of TKIs [Imatinib (range 40-5000 nM), Dasatinib (range 0.8-100 nM) and Nilotinib (range 15-2000 nM)] on T cell proliferation (by CFSE flow cytometry analysis) and function (by INFg ELISPOT assay and the expression of leukocyte activation marker CD69). In particular, T cells were obtained by OKT3/CD28 stimulation of PBMCs from both healthy donors (HD) and CML patients currently treated with standard dose of Dasatinib. *Results.* We demonstrated that among all three main TKIs used in therapy, Dasatinib inhibits T cell proliferation in a dose dependent manner in 10 out of 10 treated T cell lines isolated from HD. Indeed, cell proliferation was significantly inhibited by 10 nM of Dasatinib, 5 µM Imatinib and 5µM of Nilotinib. At the same time, Dasatinib inhibits also T cell functionality, since we demonstrated a reduction of INFg production when CTLs were stimulated by specific peptides in the presence of 25nM of Dasatinib (105±5 spot forming cells/10^5 T cells) respect to untreated CTLs (660±5 spot forming cells/10^5 T cells). Moreover, we detected a significant down-regulation of the activation marker CD69 when T cells were stimulated by OKT3 in the presence of 10nM of Dasatinib (5±2%) respect to T cells stimulated in the absence of TKI (87±3%) or in the presence of Imatinib (85±1%) or Nilotinib (86±2%). Interestingly, we observed a significant lower level of OKT3 T cell activation when PBMCs were derived from eight CML patients under Dasatinib treatment (16±10% CD69+CD3+ cells) respect to T cells derived from four HDs (87±3% CD69+CD3+ cells). In contrast, Imatinib and Nilotinib did not affect T cell function or peptide-specific CTLs at the indicated dose range. *Conclusions.* Our data demonstrated that TKIs may interfere with *in vitro* expansion of T cells and may have an immune modulating effect.

PO-029

FOLLOW UP A LUNGO TERMINE DEI PAZIENTI CON LMC-PH+ IN RISPOSTA CITOGENETICA COMPLETA CON TERAPIA INCLUDENTE INTERFERONE NELL'ERA IMATINIB - PROTOCOLLO GIMEMA CML0509

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Introduction. Interferon alpha (INF) was introduced in the treatment of Ph+ chronic myeloid leukaemia (Ph+ CML) at the beginning of the eighties and used, alone or in combination with Ara-C, until 2001, when it was replaced by Imatinib. INF frontline in CML patients in chronic phase (CP) was the first drug able to induce not only hematologic remissions, but also partial or, in some cases, complete cytogenetic responses (CCgR), and a significant prolongation of survival. In Europe, for patients achieving a CCgR with INF , Bonifazi et al reported a 10 years survival of 80% (Blood 2001). *Methods.* In this study, we thought to update the follow up of patients who were treated in Italy between 1986 and 2001 with INF -based therapy and who obtained a CCgR at least once. The aim of this study was to achieve clinical and biological information on these fascinating elite of patients during the Imatinib era, by collecting the data of haematological, cytogenetic and molecular response and by reporting the clinical events correlated with overall and progression free survival. *Results.* We collected 116 Ph+ CML patients in CP treated with INF -based therapy from 1986 to 2000 and who obtained a CCgR at least once. Among the characteristics of these patients at diagnosis, it has to be noticed that the median age was 43 years and that most of them (72%) had a low-risk Sokal. They received INF alone in 61% of the cases and an INF dose ranging between 5 and 9 MU/day in 66% of cases. The median time from diagnosis to INF start was 1 month (1 – 20), while the time from first INF dose to first CCgR was 14 months (range 3 – 97). Out of these 116 patients: - 22 (19%) remained on INF [18 (82%) maintained a CCgR but 2 (11%) died without progression; 4 (8%) lost CCgR and died in blastic phase]; - 20 (17%) discontinued INF [19 (95%) are still in CCgR without any other treatment and 1 died without progression]; - 74 (64%) were switched to Imatinib [48 (65%) because of CCgR loss; out of them 39 (81%) are in CCgR , 3 (6%) died in blastic phase, 6 (12%) died without progression. Twenty-six (35%) switched to Imatinib when they were still in CCgR and all of them (100%) are in CCgR]. The BCR-ABL transcript levels on the IS was measured in 50/99 living patients, and 17/50 (34%) were in MMR (or MR 3.0) and 33/50 (66%) were in MR 4.0 or MR 5.0. *Conclusions.* The study reports on the long term outcome of patients selected for having achieved a CCgR with INF -based therapy. There is a small number of patients treated with INF who are alive and well after 25 years from diagnosis. Optimal responders to INF are optimal responders to Imatinib. The benefit seems to be limited to low-risk patients and molecular negativity seems to be not strictly required for the long survival of these patients. Work supported by European LeukemiaNet and Cofin 2009

PO-030

BLAST CRISIS (BC) IN PATIENTS WITH PH+ CHRONIC MYELOID LEUKEMIA (CML) IN THE IMATINIB ERA: CLINICAL AND BIOLOGICAL FEATURES

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Introduction. Although Imatinib has dramatically changed the treatment of CML, and despite the improved knowledge of its possible biological and molecular mechanisms, blast transformation continues to be a potential phase in the clinical course of CML. Most data on the use of Imatinib in CML are based on results of the IRIS study: estimated EFS at 8 years was 81% and freedom from progression to Accelerated Phase (AP)/BC was 92%; only 3% of patients who achieved complete cytogenetic response (CCyR) progressed to AP/BC and none of the patients with documented major molecular response at 12 months progressed to AP/BC. *Methods.* From January 2002 to January 2012, among 130 patients with chronic phase Ph+CML (CP) who received Imatinib as first line therapy, 9 patients (7%) developed BC; none of them had received prior therapies including Interferon before starting Imatinib. We report our experience related to clinical and biologic features of this subset of patients. *Results.* Sokal risk evaluation at the time of diagnosis of CML showed that 7 patients were high risk and 2 patients were intermediate risk; all patients displayed the classic t(9;22) Ph chromosome according to standard cytogenetics; the BCR/ABL transcript at RT-PCR was b3a2 in 6 patients and b2a2 in 3 patients. The median duration of CP was 23 months (range 5-80), and the median duration of Imatinib therapy before BC was 20.5 months (range 4-78). The median time to best Cytogenetic Response (CyR), including minimal, minor, partial, complete CyR, was 12 months (range 3-32), and the median time from best CyR to BC was 8 months (range 2-67). Median age at the time of BC was 53.5 years

(range 30-93). Morphologic and flow cytometry characterization of BC revealed a myeloid subtype in 7 patients and lymphoid subtype in 2 patients. The onset of BC was sudden in one patient. Acute transformation was associated with a sudden drop of platelet counts in 6 patients; no alterations in red blood cells were detected in any patient; hyperleukocytosis was detected in 6 patients and splenomegaly in 2 patients. At standard cytogenetics at the onset of BC, one patient displayed an additional chromosomal aberration, namely chromosome 3 deletion. Five patients were screened for mutations, that were found in 2 of them (M318T, E255K). At the time of this report, after a follow-up of 63 months, 3 patients are alive (2 patients after HSCT) and 6 patients, not eligible for HSCT, died of disease progression; overall median survival was 7 months. *Conclusions.* BC is a rare but possible lethal development in patients with CML; our single center experience is in agreement with IRIS data, after 8 years follow-up, on the rate of progression to AP/CB. Further clinical and biological features should be considered at diagnosis in order to identify more appropriate treatment for patients in an era in which second generation TKIs can be used as first line therapy in patients with Chronic Phase Ph+CML.

PO-031

INVOLVEMENT OF HEME OXYGENASE 1 IN THE MECHANISMS OF RESISTANCE TO IMATINIB MESYLATE IN CHRONIC MYELOID LEUKEMIA CELLS

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Identification of imatinib mesylate (IM) as a potent inhibitor of the Abl kinase and the subsequent findings that this compound displays growth inhibitory and pro-apoptotic effects in Bcr-Abl+ cells has deeply conditioned Chronic myeloid leukemia (CML) treatment. Unfortunately the initial striking efficacy of this drug has been overshadowed by the development of clinical resistance. A wide variety of molecular mechanisms can underlie resistance mechanisms. In the recent years, heme oxygenase-1 (HO-1) expression has been reported as an important protective endogenous mechanism against physical, chemical and biological stress and this cytoprotective role has already been demonstrated for several solid tumors and acute leukemia. To investigate the effect of HO-1 expression on cell proliferation and apoptosis in chronic myeloid leukemia cells, K562 and LAMA-84 cell lines were incubated for 24h with IM (1 M) or in combination with an inducer or inhibitor of HO-1 (Hemin and SnMP). In addition, cells were also treated with HO byproducts, bilirubin and carbon monoxide (CO), or with a protease inhibitor (Ed64). After pharmacological treatments we evaluated cell viability and HO-1 expression by real-time PCR. Pharmacological induction of HO-1 was able to overcome the effect of imatinib. The cytoprotective effect of HO-1 was further confirmed after silencing HO-1 by siRNA. Interestingly, neither bilirubin nor CO were able to protect cells from IM-induced toxicity. The protective effect of HO-1 was mitigated by the addition of Ed64, preventing HO-1 nuclear translocation. We also analyzed 96 kinase genes using TaqMan® Low Density Array Human Kinases Panel and found that induction of HO-1 in combination with IM increased seven different kinases. Finally, IM was able to increase the formation of cellular reactive oxygen species. This effect was reversed by HO-1 induction or the addition of N-acetylcisteine. In conclusion, the protective effect of HO-1 on IM-induced cytotoxicity does not involve its enzymatic by-products, but rather the nuclear translocation of HO-1 following proteolytic cleavage. Migration of HO-1 into the nucleus activates several kinases which may be responsible for mitogenic signals activation.

PO-032

IDENTIFICATION OF GENES INVOLVED IN LYSOSOME MEDIATED TYROSINE KINASES RECYCLING IN THE PATHOGENESIS OF CHRONIC MYELOID LEUKEMIA

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Introduction. The role of Bcr-Abl in the pathogenesis of Chronic Myeloid Leukemia (CML) is well established, however the mechanisms involved in progression remain poorly understood. By making use of our Drosophila Melanogaster transgenic model for human Bcr-Abl gene, we have identified the involvement in CML progression of different genes regulating the recycling and the degradation of tyrosine kinase (TK) receptors through the assembly of clatrin coated vesicles. Among them, Rab5 gene is involved in lysosome mediated protein recycling and many TK receptors are switched off through this mechanism. *Methods.* Rab5 expression was measured by quantitative RT-PCR in 90 samples from 80 CML patients (32 PB and 58 BM). Among those, 53 samples were collected at diagnosis, 9 at the time of Imatinib resistance in Chronic Phase (CP) patients, 7 in Accelerated Phase and 11 in Blast Crisis. Rab5 expression was also analyzed in 14 patients during disease remission, while 28 healthy donors (7 PB and 21 BM) were evaluated as controls. RAB5 protein expression was investigated by Western Blot (WB) and Immunofluorescence (IF) analyses. Furthermore, 15 CP CML cases and 9 healthy donors (2 PB and 7 BM) were evaluated for Dynamin 1-like (DNM1L) expression. We also selected Syntaxin 5 (STX5), Synaptotagmin I (SYT1), Kinesin family member 14 (KIF14), EFR3 homolog B (EFR3B), Bridging integrator 1 (BIN1), Dynein light chain (DYNLRB2), Vacuolar Protein Sorting 41 (VPS41), Guanine Nucleotide Binding Protein gamma 7 (GNG7), as genes involved in vesicle recycling. *Results.* We found that in CML patients Rab5 expression was significantly decreased in either BM or PB ($P<0.001$ and $P<0.0001$) compared to healthy subjects. At the time of remission, the transcript levels returned to normal values. Similarly, Dynamin 1-like expression was significantly downregulated in CML compared to healthy donors ($P<0.004$). The RAB5 protein level, analyzed by WB and IF, demonstrated a concordance with the mRNA levels. *Conclusions.* Although preliminary, our results show a significant decrease of gene expression and proteins involved in the assembly of clatrin coated vesicles and lysosome mediated protein recycling. We are performing further gene expression and protein analyses on the other 8 selected genes involved in vesicle recycling. These data may suggest an additional mechanism responsible for the maintenance of BCR-ABL signalling through a defect in the degradation of TK receptors including BCR-ABL itself.

PO-033

ERK5-INHIBITION AS A NOVEL APPROACH TO TARGET CML STEM CELLS

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Introduction. Evidences obtained in our laboratory indicated that the leukaemia stem cell (LSC) potential of chronic myeloid leukaemia (CML) cell populations is resistant to, and selected in, severe hypoxia (0.1-1% oxygen). The Extracellular signal-Regulated Kinase 5 (ERK5) is a member of the mitogen-activated protein kinase family involved in the control of cell survival and proliferation and in the pathogenesis of different types of cancer, including CML. The main target of this study was to address the effects of ERK5 inhibition on the maintenance of hypoxia-selected LSC of CML by the Culture-Repopulating Ability (CRA) assay. *Methods.* The K562 and KCL-22 human stabilized CML cell lines, where ERK5 is constitutively activated, were used both for all the experiments. Cells were incubated in hypoxic (~0.1% O2) or normoxic (used as control) primary cultures (LC1) in the absence or the presence of MEK5 or ERK5 inhibitors. Cells were harvested at different times and analysed. Cell survival, cycling, proliferation and apoptosis were assessed by counting Trypan blue-negative cells or flow cytometry using propidium iodide (PI) and anti-annexin-V antiboidies. At day 7 of incubation, LC1 cells were transferred to non-selective normoxic secondary cultures (LC2) of CRA assays in the absence of inhibitors, to evaluate LC2 repopulation. *Results.* Hypoxia prevents the cell number increase which occurred in normoxia and determined early and massive apoptosis, as well as cell cycle arrest of surviving cells. Consistently, the AKT protein, an important pro-survival signal, was cleaved in hypoxia. Hypoxia decreased the intensity and duration of ERK1/2, p38 and JNK phosphorylation/ activation occurring in normoxia, without altering the expression of these proteins. On the other hand, hypoxia suppressed ERK5 constitutive activation and protein expression, unchanged in normoxia, but not ERK5 mRNA. The effects of the inhibition of the MEK5>ERK5 pathway were then determined. We tested several MEK5>ERK5 pathway inhibitors and identified one specific ERK5 inhibitor that completely suppressed LSC maintenance in hypoxia. In LC1, this inhibition resulted in a significant increase of the percentage of cells in G0/G1 and in a modest reduction of apoptosis, pointing to a cytostatic, rather than cytotoxic, effect of ERK5 inhibition. *Conclusions.* The maintenance of leukaemia stem cells of CML is impaired by ERK5 inhibition, which therefore emerges as a potential novel strategy for CML therapy.

PO-034

HIGH BCR-ABLIS EXPRESSION AT DIAGNOSIS AND AFTER 3 AND 6 MONTHS OF TREATMENT ARE ASSOCIATED WITH UNFAVORABLE RESPONSES TO IMATINIB

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Introduction. Imatinib mesylate (IM) has shown remarkable efficacy for the treatment of Chronic Myeloid Leukemia (CML) patients (pts) in the chronic phase of the disease. However, a growing number of pts either fail IM or develop intolerance to the drug. We wanted to identify biological parameters predictive of IM response (at diagnosis or during the first months of therapy) in order to recognize pts with a more aggressive disease that should receive alternative drugs. *Methods.* We examined the outcomes of the first 230 CML pts accrued to the observational SCREEN (Sicily and Calabria CML REgional ENterprise) multicenter study, recruiting newly-diagnosed CML pts receiving IM 400 mg daily. Median follow-up was 42 months (range 12-72). Complete hematological (CHR), cytogenetic (CCyR) and major molecular responses (MMR) were rated according to the 2009 European Leukemia Net guidelines. Peripheral blood samples were used for BCR-ABL determination by quantitative real-time polymerase chain reaction according to the International standardized Scale (IS) using either GUS (at diagnosis) or ABL (at every other time-point) as reference genes. Pts were stratified according to clinical and molecular responses or BCR-ABL transcript levels at diagnosis and analyzed for their outcome on an intention to treat basis. *Results.* At 12 months, cumulative incidences of CHRs, CCyRs and MMRs were 97.1%, 60.7% and 10.8%. At 60 months, incidences of CCyR and MMR increased to 82.3% and 73.8%, respectively. According to the ELN criteria, 111 pts (48.3%) achieved an optimal response; 63 pts (27.4%) had a suboptimal response; 46 pts (20%) failed IM because of either primary (30 pts) or secondary (16 pts) resistance. Only 10 pts (4.3%) were intolerant to IM. Kaplan-Meyer estimates for overall, progression-free, event-free (EFS) and failure-free survival (FFS) at 60 months were 93.8%, 97.8%, 88% and 76%. When we clustered all subjects in optimal responders (ORs) and suboptimal/resistant (S/R) pts and correlated response to therapy with various molecular characteristics we found that high BCR-ABLIS transcripts at diagnosis (using GUS as a reference gene) predicted response to IM ($P<0.0001$). Moreover, high BCR-ABL/GUSIS levels at diagnosis significantly correlated with lower rates of CCyR ($P<0.0001$), EFS ($P<0.0004$) and FFS ($P<0.0001$). We also observed that pts displaying >10% BCR-ABL/ABLIS after 3 months of IM or >1% BCR-ABL/ABLIS after 6 months of therapy had a significantly lower probability of achieving a CCyR and an optimal response ($P<0.0002$ and $P<0.0001$, respectively). *Conclusions.* High levels of BCR-ABL/GUSIS transcripts at diagnosis allow the rapid identification of CML pts that are unlikely to benefit from IM. Furthermore, failing to achieve BCR-ABL/ABLIS transcripts <10% after 3 months or <1% after 6 months of IM significantly reduces the probability of subsequently obtaining a CCyR and an optimal response.

PO-035

CIRCULATING ENDOTHELIAL CELLS AS A BIOMARKER OF ENDOTHELIAL DAMAGE IN PATIENTS UNDERGOING ALLOGENEIC STEM CELLS TRANSPLANTATION: CORRELATION WITH GVHD ONSET

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Introduction. AlloBMT can be burdened by life-threatening complications, being GVHD the major cause of morbility and mortality. Clinical and physio-pathological evidences have shown that vascular endothelium could be a target of GVHD in very early phase; therefore markers of endothelial damage are warranted as valuable support in GVHD diagnosis. Several soluble factors have been tested as endothelial injury biomarkers, but any proved sufficient specificity and reliability to enter clinical practice. We conducted a study with primary endpoint to identify and count CEC in peripheral blood of patients undergoing alloBMT as a function of endothelial damage. *Methods.* The CellSearch System® was used to capture and enumerate CEC. Magnetic particles conjugated to anti-CD-146 are used to capture CEC from 4.0 ml of blood. Enriched cells are stained with DAPI and anti-CD-105-PE antibody. APC conjugated anti-CD-45 is used to exclude leukocytes. Enriched and stained cells are dispensed into a MagNest® cartridge for magnetic mounting. The cartridge is scanned and individual images of cells are presented for review and scored as CEC, based on CD146+, CD105+, DAPI+ and CD45- phenotype and cell morphology. Patients undergoing alloBMT were tested before, after the conditioning regimen and at weekly intervals till day +100 from alloBMT. Ten healthy subjects have served as controls. *Results.* We enrolled 20 patients with hematologic neoplastic diseases (5 HD, 5 AML, 3 ALL, 4 MM, 1 CLL, 1 CML, 1 SAA) undergoing alloBMT from either HLA-matched familial (n=8) or unrelated donor (n=12). The median CEC/ml pre-alloBMT was 19 (n=20, range 4-94), going up to 30 CEC/ml (n=19, range 6-648, P=NS) at the end of the conditioning regimen. At GVHD onset the median CEC/ml was 58 (n=6, range 32-210, P=0.004), being already increased at evaluation performed one week earlier (n=6, 47 CEC/ml, range 26-56, P=0.003). At GVHD response the median CEC/ml decreased to pre-alloBMT values (n=6, 22 CEC/mL, range 6-86, P=NS). The median count in the 10 healthy subjects was 2 CEC/mL (range 1-14). *Conclusions.* Circulating endothelial cells can represent a promising marker to monitor endothelial damage in patients undergoing alloBMT. We have showed a statistical significant increase in CEC numbers at GVHD onset, but also one week earlier, with a normalization at treatment response. However, our results need to be confirmed in a larger series of patients. The confirmation of the clinical utility of CEC counts, together with the use of a semi-automatic, standardized and reproducible technology, could allow a valuable help in the diagnostic definition of GVHD in early phase, an information on the response to treatment and moreover could be a valid complement in the prognostic stratification of patients candidates to alloBMT.

PO-036

RECONSTITUTION OF THE T CELL COMPARTMENT AND THE THYMIC FUNCTION AFTER REDUCED-INTENSITY ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Background. The thymus plays a pivotal role in generating naive T cells from hematopoietic precursors and long-term immune reconstitution after allografting. *Aims*. To asses the short- and long-term reconstitution of the CD4+ T cell compartment and the residual thymic activity in elderly patients undergoing allogeneic hematopoietic cell transplantation. *Methods.* The study population consisted of 63 consecutive recipients, median age 56 (r 23-67), of a T cell repleted allograft from siblings or unrelated donors after thiotepa/cyclophosphamide-based reduced-intensity or low-dose total body irradiation-based non-myeloablative conditionings. One patient underwent thymectomy 10 years before the allograft. The reconstitution of the CD4+ T cell compartment was evaluated by flow cytometry. Naïve, central memory, effector memory, and "revertant" CD4+ T cells were identified by co-expression of CD45RA and CD27, CD45RO and CD27, expression of CD45RO (CD27 negative), and co-expression of CD45RA and CDRO, respectively. Blood samples were drawn at baseline, and at 3, 6, 12, 18 and 24 months post-transplant. To quantify residual thymic function and to differ true naïve CD4+ from "revertant" CD4+ T cells, T-cell receptor excision circles (sjTRECs) were assessed by real time PCR at the same time points on DNA extracted from sorted CD4+ T cells. sjTRECs are episomal excision products of the T cell receptor gene rearrangements that occur in maturing thymocytes; do not replicate during mitosis and are diluted during cell division providing an excellent measure of thymic function during periods of immune reconstitution. Primary endpoint was the trend over time of sjTRECs copy number/ 100 ng DNA; secondary endpoints were trends over time of naïve, central memory, effector memory, and revertant CD4+ T cell numbers after transplant, and their potential modifications by patient- and transplant-related independent variables. Univariate and multivariate analyses included: age at transplant (>56 vs. ≤56 years), gender (female vs. male), conditioning regimen (reduced-intensity vs. non-myeloablative), donor type (unrelated donor vs. sibling donor), disease status at transplant (less than CR vs. CR), occurrence of acute graft-vs.-host disease (GVHD) and chronic GVHD, number of CD3+ and CD34+ cells/ kg recipient body weight infused. *Results.* Naïve, central memory, effector memory, and, to a far lesser extent, "revertant" CD4+ T cell values showed a gradual significant increase throughout the study period. However these values were lower than those observed in healthy donors. A gradual statistically significant increase in sjTRECs from baseline up to 2 years post-transplant was also observed (P<0,001). Importantly, siTRECs levels were undetectable in the patient who had undergo thymectomy prior to transplant. By multivariate analysis, a significantly lower sjTRECs increase was seen in patients conditioned with a reduced-intensity rather than a non-myeloablative regimen (P=0,008), transplanted from an unrelated donor (P=0,002), and in those who developed chronic GVHD (P=0,029). By contrast, no differences were seen in patients who developed acute GVHD from those who did not. *Conclusions.* Effective residual thymic activity contributing to long-term immune reconstitution was seen in this elderly patient cohort. The thymus remains sensitive to chemotherapy-induced damage and is the target of chronic GVHD but not acute GVHD.

PO-037

DEFICIENCY OF LONG PENTRAXIN 3 (PTX3) IN BONE MARROW-DERIVED MESENCHYMAL STEM CELLS IMPAIRS WOUND HEALING IN MICE

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Introduction. Although several studies have shown the capacity of mesenchymal stem cells (MSCs) to repair and regenerate different tissues, the mechanisms underlying these processes are not understood. In the present study we analyzed the role of the long pentraxin 3 (PTX3) in enhancing the wound closure. PTX3 is a multifunctional protein produced by MSCs after activation with inflammatory cytokines, which is involved in innate immunity, inflammation and extracellular matrix deposition. *Methods.* PTX3-/-MSCs were collected from bone marrow of PTX3 knockout mice. After 3-5 culture passages the expression of surface markers was analyzed by flow cytometry and their osteogenic and adipogenic differentiation capacity was detected by alizarin red O and oil red S staining, respectively. The ability of PTX3-/-MSCs to abrogate T cell proliferation was evaluated by co-culturing of MSCs and PBMCs previously activated with Phytohaemagglutinin (PHA). Finally, equal number of both PTX3-/-MSCs and WT MSCs were implanted into excisional wounds created by a biopsy punch on the back of allogenic WT and PTX3-/- mice. Wound area was measured up to 14 day and calculated using an image analysis program. The wound specimens were col-

lected at 2, 7 and 14 days and processed for istological analysis. *Results.* By analyzing MSCs obtained from bone marrow of PTX3 knockout mice we demonstrated that, similarly to MSCs obtained from wild type mice (WT MSCs), PTX3-/-MSCs displayed typical fibroblastoid morphology, they were consistently devoid of contaminating hematopoietic cells and expressed common MSC markers. In addition, these cells were able to differentiate into adipocytes and osteoblasts, and drastically decreased the mitogen-induced proliferation of lymphocyte, in a dose dependent manner. Importantly, in a mouse model of wound healing, PTX3-/- MSCs showed an highly significant defect in wound closure compared to WT MSCs at each time point. Histologic evaluation of skin samples from mice treated with PTX3-/-MSCs showed a reduction in the granulation tissue, a significant increase of polymorphonuclear (GR-1+) cells in the wound bed and an enhancement of the fibrin deposition at the 2nd day after injury. Accordingly, PTX3-/- MSCs also failed the ulcers closure in PTX3 knockout mice. *Conclusions.* we demonstrated that PTX3 deficiency does not alter the phenotype or the capacity of MSCs to differentiate into mesengetic lineages; of relevance, the production of PTX3 represents an essential requirement for MSC ability of enhancing tissue repair.

PO-038

IMPACT OF MYELOID DERIVED SUPPRESSOR CELLS (MDSC) FREQUENCIES IN G-CSF MOBILIZED UNRELATED DONOR GRAFTS ON THE INCIDENCE OF ACUTE GVHD AFTER ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Background. Recent evidence suggests that graft-versus-host disease (GvHD) can be abrogated by ex vivo expanded, bone marrow derived, myeloid-derived suppressor cells (MDSCs) generated in the presence of GM-CSF, G-CSF and IL-13 in mice (Highfill et al. Blood 2010). Whether MDSCs play a role in human allogeneic hematopoietic stem cell transplantation (allo-HSCT) is still unclear. We hypothesized that G-CSF stem cell mobilization may be protective from acute GvHD (aGvHD) in unrelated donor HSCT by increasing MDSC frequencies. *Methods.* G-CSF-mobilized peripheral blood (PB) samples were collected from 40 healthy unrelated donors (median age 34, range 20-43, male/female 31/9) who received G-CSF (Filgrastim) at 10 g/kg/day for 5 days. Donor selection had been performed according to standard criteria, including molecular typing for HLA-A,-B,-C, DRB1, and DQB1. Donors were 10/10 HLA-matched (MUD) in 20 cases, 9/10 in 13 cases and the remaining 7 cases were mismatched in 2 or more HLA loci (MMUD). Patients (median age 46, range 18-67) received reduced intensity conditioning based or low-dose total body irradiation (TBI 2Gy) (8), Fludarabine-Rabbit Antithymocyte Globulin (rATG) (9) or Thiotepa-rATG (23). Diagnosis were lymphomas (29), myelomas (8), acute myeloid leukemia (3). GvHD prophylaxis was cyclosporine plus either methotrexate (36) or mycophenolate mofetil (4). As controls, PB samples were collected from 10 healthy adults. Informed consent was obtained from all subjects. Cells were characterized using flow cytometry with Abs against CD3;CD14;CD16;CD19;CD20;CD56;CD11b;HLADR;CD33 after Ficoll separation. The frequencies of MDSCs in the graft were correlated with the clinical characteristics and outcome of the 40 patients. *Results.* Expansion of MDSCs defined as Lin-/LoHLADR-CD33+CD11b+ in the PB of G-CSF-treated unrelated donors was found with respect to steady state control individuals (P<0.03, Mann Whitney-U). Acute GvHD occurred in 16 of 40 patients (40%). There was no significant correlation between the incidence of aGvHD and the degree of HLA incompatibility or the presence of donor-recipient sex mismatches. Neither the conditioning regimens nor the GvHD prophylaxis had effect on risk of aGvHD. Conversely, aGvHD patients received grafts containing significantly lower number of MDSCs when compared to non- aGvHD patients (P<0.006, Mann Whitney-U). The ability of MDSC levels in the graft to predict the occurrence of aGVHD was determined by the receiver operating characteristic (ROC) curve: sensitivity was 100%, specificity 60%, AUC=0.768. The immunosuppressive activity of MDSC on activated T lymphocytes was also confirmed. *Conclusions.* G-CSF mobilization significantly increases circulating MDSC in Matched Unrelated Donors. A significant correlation between the frequencies of MDSC present in the graft and the incidence of aGvHD was found. This finding should be confirmed in prospective studies and might be relevant to tailored GvHD prophylaxis.

PO-039

HLA-TYPING BY PYROSEQUENCING TECHNOLOGY: A NEW PROMISING QUICKLY AND ACCURATE APPROACH WITHOUT PHASE AMBIGUITIES

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Introduction. The HLA region on chromosome 6p is characterized by an high degree of heterogeneity and this is translate into a high number of alleles and aplotypes. The techniques of HLA-genotyping have evolved during the last 30 years, but in some cases is difficult determine the right call for each HLA-locus because there are ambiguities. Ambiguities derived from the incomplete genomic coverage or the contemporary Sanger sequencing of two eterozygous alleles that determines different aplotypes (for Phase ambiguities). The new generation sequencing technologies have the potential to perform HLA-typing in a rapid and accurate way without Phase ambiguities. *Methods.* To perform high-resolution HLA-typing we used a custom assay who combine a two PCR plates with 2 run of pyrosequencing (454 GS Junior) and subsequent bioinformatic analysis. The output file is then uploaded into the Assign ATF software to align all sequences with the reference database (ref 3.2.0 2010). *Results.* Using the method of Multiplex Identifier (MID) tag, we can pooling amplicons from different samples; in this case we have pooled 10 different samples. The PCR reaction generates 140 amplicons who correspond to exons 2, 3 and 4 of HLA-A/B/C, exons 2 and 3 of DQB1and exon 2 of DPB1, DQA1, DRB1/3/4/5. Then the total number of amplicons must be splitted into 2 run of GS Junior sequencing. We have obtained a mean of 1001 reads for each amplicon. The assignment of unambiguous genotype was possible in 79% of cases. The ambiguities are linked to the assay design, in fact for HLA-class II we can study only exon 2, but to resolve certain ambiguities we must indagate at least the exon 3. The rare ambiguities on the locus B and C have a little biological importance because both the alleles coding for the same peptide binding domain, instead the genomic differences between the two alleles are located on the transmembrane domain coding region. All cases analyzed in this study were genotyped using conventional strategies, for the most part ssp; the concordance between our results and precedent is 100%. We have also investigate the allele balance in terms of number of reads that correspond to each allele on a locus. The mean allele ratio is 1,2 [1-6] and shows a good allele sharing. *Conclusions.* Using a NGS technique we have performed high-resolution HLA-typing for the most locus of the samples, quickly and without phase ambiguity. The method is very accurate and ensure allelic resolution for the locus that have major clinical importance. The system discriminates very well the alleles that determines differences on the peptide binding domain. In conclusion this is an hopeful way to perform an important test like HLA-typing. This work was supported by grant of BCC Pompiano e Franciacorta and by Roche.

PO-040

CELL-THERAPY WITH HLA-MISMATCHED MOBILIZED PERIPHERAL BLOOD STEM CELLS AFTER STANDARD CHEMOTHERAPY IN ELDERLY PATIENTS WITH ACUTE MYELOID LEUKEMIA: PRELIMINARY DATA ON FEASIBILITY AND SAFETY

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Introduction. Effective therapeutic options for acute myeloid leukemia (AML) in elderly patients are still lacking. An innovative approach,

including the infusion of mobilized peripheral blood stem cells (PBSC) from haploidentical sibling donors after induction chemotherapy, has been recently described (Guo *et al*., Blood 2011). The addition of haploidentical PBSC apparently improves the outcome of elderly AML patients. So far, these data have not been confirmed by other groups worldwide. *Methods.* Three elderly patients, two with AML secondary to myelodysplasia and one with therapy-related AML (t-AML), were treated with the PBSC-based program. Median age was 66 years (64-76 years), one patient (t-AML) had a mixed lymphoid-myeloid phenotype and complex karyotype. Patients received the originally described regimen, including mitoxantrone 8 mg/sm for 3 days and Ara-C 150 mg/sm for 7 days, followed on day 9 by the infusion of G-CSF mobilized PBSC from haploidentical donors, with a threshold of >0.5x10^6 CD34+ cells/kg and <1x10^8 CD3+ cells/kg. Mixed lymphocyte reactions were performed and results confirmed the potential of patient T-cells to reject their haploidentical counterparts. Results: The therapy was well tolerated, with moderate fever for 3-7 days after PBSC infusion in all 3 patients and a moderate bilirubin increase in one patient. One patient developed infective pneumonia during aplasia, which resolved after treatment with liposomal amphotericin B. None of the patients developed acute GVHD. As of May 2012, follow up data are available for one patient, while two patients are still on treatment. Patient #1 had a t-AML secondary to 6 lines of chemotherapy received for ovarian cancer, with stable ovarian cancer under tamoxifen. After induction chemotherapy followed by the infusion of haploidentical PBSC, a bone marrow aspirate showed blasts reduction from 90% to 45%. Considering the refractoriness to chemotherapy, the patient received an intensified salvage split regimen (high-dose Ara-C and idarubicin plus cyclosporine to overcome MDR resistance), followed again by a second infusion of haploidentical PBSC at the same cell dose of the first cycle. After this cycle, blood cell counts progressively improved and a bone marrow aspirate showed complete remission of disease with no detectable blasts at flow cytometry. Donor microchimerism was observed after each infusion, peaking at day 14. At last follow-up the ovarian cancer was in mild progression and the patient is now being treated with progesterone acetate (figure 1). A consolidation regimen with the third infusion of PBSC is planned. Conclusions: This preliminary report attests the feasibility and safety of the infusion of haploidentical G-CSF mobilized PBSC as part of treatment for elderly AML patients with poor prognostic features. The prolonged follow-up on large patients series will define the real efficacy of this innovative cell-therapy based approach.

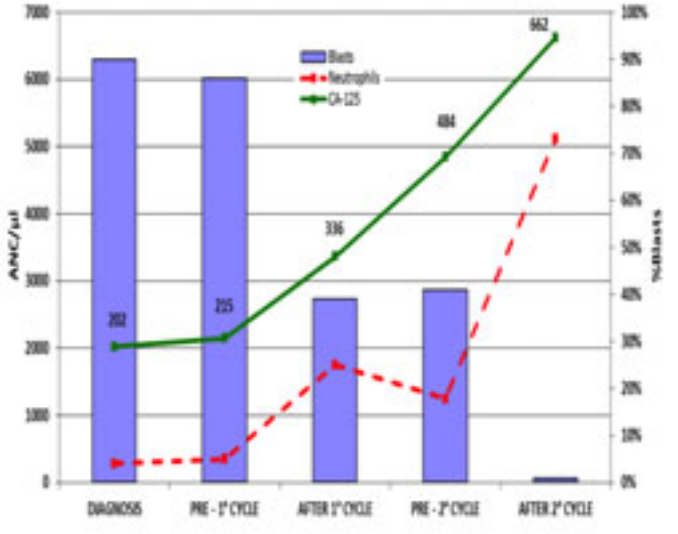

Figure 1.

PO-041

IMPACT OF CD3/T REGS RATIO IN DONOR GRAFT ON OVERALL AND RELAPSE-FREE SURVIVAL RATES IN ALLOGENEIC PERIPHERAL BLOOD STEM CELL TRANSPLANTATION

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Introduction. The therapeutic efficacy of allogeneic stem cell transplantation (alloSCT) for hematological malignancies relies largely on the graft versus leukemia (GvL) effect exerted by the donor CD3 cells, but an uncontrolled graft-versus-host-disease (GvHD) bears a risk of complications. On the other hand, T regs cells (CD4+CD25high Foxp3+) are believed to maintain tolerance and to inhibit GvHD after alloSCT; also, the Foxp3 gene encodes a transcription factor that is a key for thymic development, so T regs cells could preserve an optimal microenviroment for the reconstitution of functional immunity after alloSCT. Moreover, when looking at allotransplant patients' survival rates, while it is largely known the impact of acute GVHD (triggered by CD3 donor T cells) on survival, there is no evidence that donor graft CD3/T regs ratio may give an effect in terms of overall and relapse free survival rates. *Patients and methods.* In this study we analyzed the graft CD3+/Tregs ratio (gCD3/Tregs R) and determined its impact on acute GVHD (aGVHD), immunological recovery and survival rates (OS and RFS) after myeloablative alloPBSCT. We analyzed 75 consecutive patients transplanted with unmanipulated peripheral blood stem cells from an HLA identical related donor (n=50) or an HLA identical unrelated donor (n=25); diagnoses were acute myeloid leukaemia (n=62), acute lymphoblastic leukaemia (n=13). The median CD3+ and Tregs dose administered was 238 (range (r): 67-550) and 12,5x10^6/Kg (r: 2-21), respectively; the median gCD3/Tregs R was 19 (r: 8-250). Patients were subdivided into a high gCD3/Tregs R (>=36) group (HR group n= 31) and a low gCD3/Tregs R (<36) (LR group n=44). *Results.* The incidence of aGVHD (grade II-IV) in the low gCD3/Tregs R (LR) group was lower than in the high gCD3/Tregs R (HR) group (9/44 or 20% vs 24/31 or 77%, P<.001). At multivariate logistic regression, gCD3/Tregs R was correlated both with aGVHD (Odds Ratio (OR): 2.60, 95% CI (1.35,4.90), P=.05) and with CMV infection/disease (OR: 2.45, 95% CI (0.8,5.50), P=.05). The OS rate at 5 years was significantly affected by the number of transplanted T regs (66 vs 30% in the L and H gCD3/Tregs R group, respectively, P<.001); on the contrary, RFS rate was not affected by gCD3/Tregs R (P=ns). *Conclusion.* Taken together, our data may suggest that Tregs content is able to mediate protective effects against aGVHD, while preserving GvL effects as demonstrated by RFS comparison between H and LR groups. However, a longer follow-up after alloSCT is needed to understand the real contribution of gCD3/Tregs R on survival rates.

PO-042

FEASIBILITY AND OUTCOME OF AUTOLOGOUS STEM CELL TRANSPLANT (ASCT) IN ELDERLY PATIENTS: ANALYSIS OF TWO AGE RANGES

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ASCT is considered the standard of care for multiple myeloma (MM) and relapsed chemosensitive aggressive non Hodgkin lymphoma (NHL). The issue, however, is controversial in elderly patients, as in recent years the procedure has become safer due to improvement in supportive care but, on the other hand, the availability of newer drugs could reduce the need to consolidate a response with high dose therapy. In this study we retrospectively analyzed 82 consecutive patients aged > 60 who were enrolled in a transplant program in our Institution from January 2003 to March 2012. We comparatively analyzed the outcome of patients aged 60-65 (elderly but still included in most transplant protocols – group A) and that of patients aged >66 (group B). The characteristics of the patients, including M/F ratio, disease type, previous therapy, disease status at transplant, mobilization regimen and preparative regimen to transplant were well balanced in the two groups. Mobilization failure occurred in a slightly higher percentage of older patients (17.9%) than younger ones (4.6%), however the majority of these patients was subsequently rescued with a second mobilization±bone marrow harvest

(N = 4) or plerixafor (N=3). Forty-one patients in group A and 32 in group B successfully mobilized pheripheral blood stem cells (PBSC); in a median of 2 pheresys, the median number of collected CD34+ cells was 7.18 and 8.82 x 106/kg in group A and B respectively. The percentage of patients proceeding to transplant averaged 90% in both groups, a double transplant was performed in 9/24 and 8/17 MM patients in group A and B respectively. Day 100 non-relapse mortality was 5.4% (3/42 patients in group A and 1/32 in group B), hematological recovery was similar in the two groups (median = 11 days for PMN > 500/mmc and 12 days for platelets > 20000/mmc). Relapse rate was higher, though not significantly, in older patients (59.3% vs 47.6%), Median progression-free survival was 35 months in group A and 19 months in group B (P= ns), median overall survival was 58 months in group A and 38 months in group B (P= ns). Our data indicate that ASCT can be safely performed in patients aged > 66yrs yielding comparable results as compared to patients aged 60-65 yrs.

PO-043

STEM CELLS PROPERTIES IN HUMAN BONE MARROW ADIPOCYTES

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Introduction. Mature adipocytes isolated from omental and subcutaneous adipose tissue expressed stem cells and reprogramming genes, showing in culture characteristics and functions of mesenchymal stem cells (1). Here, we characterized bone marrow adipocytes (BM-A) to better understand their functions in hemopoietic microenvironment and to compare their stemness properties with adipose tissue (AT-A), despite their different anatomical site. *Methods.* Mature adipocytes isolated from healthy human bone marrow were subjected to ceiling culture, after collagenase digestion and filtration. We studied structural characteristics by electron microscopy, proliferative ability in liquid culture, molecular and immunophenotypic profile. *Results.* In culture, bone marrow adipocytes showed a morphology similar to AT-A, characterized by the presence of a single, large cytoplasmic lipid droplet that accounted for about 90% of its volume. BM-A were smaller than AT-A and, contrary to AT-A, only few cells changed their shape into a fibroblast-like cells during the culture time, maintaining their intracytoplasmatic lipid droplets. In particular, BM-A didn't lose their lineage morphology and didn't acquire the proliferation potential. Flow cytometric analysis revealed that these cells expressed cell-surface antigen profile similar to AT-A, but at lower level (CD34, CD133, CD117, CD271 and CD90). Moreover, at molecular level BM-A expressed statistical significant differences respect to AT-A for the embryonic stem cells and reprogramming genes tested. Nanog was no detectable in BM-A, while in AT-A was highly expressed, and Tbx1 and Sox17 were expressed at lower level respect to AT-A. Quantitative data for reprogramming genes showed that Oct4, Klf4 and c-myc were expressed at lower level respect to AT-A, while there was no presence of Sox2 in bone marrow fat. Furthermore, the cells analyzed had telomere significantly shorter than AT-A. *Conclusion.* Taken together, comparison of immunophenotypic and molecular expression profiles in BM-A with AT-A indicated that bone marrow fat appeared to have a gene expression pattern that distinguished them from fat cells in the adipose tissue. However, adipocytes from both depots displayed also common morphological and molecular features. The data suggested that BM-A showed different stem cells properties respect to AT-A, certainly linked to their role in the maintenance of the bone marrow microenvironment. Bone marrow fat may acquire specific functions depending on the local environmental demands.

Reference

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PO-044

A STUDY OF THE INTERACTION OF NATURAL KILLER (NK) CELLS AND AML CELLS: PRELIMINARY *IN VITRO* DATA

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Introduction. We have previously demonstrated that immunotherapy with allogeneic partially HLA mismatched NK cells may contribute to the cure of AML in patients with high risk disease (Curti A. et al,Blood 2012).However,the mechanism of the interaction between NK cells and AML cells is unknown.In this study we aimed to characterize the interaction between AML cells and NK cells *in vitro*. *Methods.* CD3-CD56+ immunomagnetically isolated NK cells were cultured with mononuclear cells (MNC) from patients with AML for 2-3 days and we evaluted as surrogate of activation, the upregulation of the activation molecules CD25 and CD69 on NK using flow cytometry. We test the ability of NK cells to induce on AML cells the expression of costimultory molecules (CD86, CD40) and adhesion molecules (CD11c). *Results.* The data have shown an up-regulation of activating molecules CD25 and CD69 after 2 and 3 days of culture with AML cells (CD25 MFI 15±10;CD69 10±8)that decrease after 4 days accordingly with litterature. We have used only NK cells that did not express the KIR ligands that could be inhibited by the HLA molecules express on CD34+ cells.Furthermore, in some but not in all experiments, we have also observed up-regulation of NK activation receptors in particular NKG2D,NKp30,NKp44 (NCRs). Furthermore, we observed that, after coculture with NK, there is an up-regulation of the adhesion molcule CD11c and of the co-stimulatory molecules CD86 and CD40 on the surface of AML cells that is comparable to what is observed when AML cells are cultured with cytokines such as GM-CSF,TNF and IL-4. *Conclusions.* These data shown that there is an interaction between NK cells and AML cellls as shown by up-regulation of activation molecules on NK cells and costimulatory molecules on AML cells.Further studies will evaluate the role of activating NK receptors in the recognition of AML cells and eventually the ability of NK cells to kill AML cells.

PO-045

IN VITRO EXPANSION OF UMBILICAL CORD MESENCHYMAL STEM CELLS : A COMPARISON AMONG DIFFERENT SERUM-FREE CULTURE MEDIA

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Introduction. Increasing interest is currently set in clinical applications of human mesenchymal stem cells derived from Wharton's jelly (UCMSC) because of potential off-the-shelf availability at cord blood banks. Foetal Bovine Serum (FBS) is routinely used for the expansion and differentiation of UCMSC; however such addictive represents a potential hazard in clinical applications, due to the risk of transmitting viral/prion disease and/or inducing xenogenic immune reactions in the recipient. For clinical purposes, it is of major importance to find a method to amplify UCMSC in a short period of time and to define conditions "serum free" to obtain standardized preparation of UCMSC. Therefore we compared the efficacy of three different culture media with "classical" medium alpha MEM containing 20% FBS: -commercial serum free medium (StemPro MSC SFM XenoFree - medium A) -medium A supplemented with human AB serum; -medium A supplemented with a serum sostitute (Ultroser-G) *Methods.* UCMSC were isolated from 5 human umbilical cords. Wharton's jelly was separated from the cord vessels and placed in four different 6-wells dishes containing: StemPro MSC SFM XenoFree - medium A (GIBCO, Invitrogen), medium A supplemented with 10% human AB serum (medium B), medium A with 4% serum substitute contains a human protein (Ultroser-G) (medium C) and medium alpha MEM containing 20% FBS (medium D). Fresh medium was added twice a week up to 90% confluence. MSC were then harvested and re-plated at 8,000 cells/cm^2 for five passages Doubling Time (DT) values were calculated according to established formulas. Osteogenic, chondrogenic and adipogenic differentiations were performed according to standard protocols and confirmed by cytofluorimetric analysis. *Results.* MSCs isolated in all media were morphologically similar and showed the same differentiation potential. These cells failed to mark with haematopoietic markers CD14,CD34,CD19 and CD45 but were positive for molecules

CD 73, CD90 and CD105 according to the mesenchymal immunophenotype. Medium C showed a lower DT (passages P0-P5) than the other media; however statistical significance was reached only with media A and D (Table1). *Conclusions.* Our observations suggest that serum-free expansion can be achieved for UCMSC, although at a lesser extent in comparison with FCS-supplemented media. Preliminar data showed that medium supplemented with Ultroser-G allowed a better expansion of UCMSC, in a shorter period of time as opposed to that of medium serum free, and the results were reproducible.

Table 1. Meandoubling time (5 cultures for each medium) of cells grown in different media (column1-4), and results of pairwise t-tests.

	A	B	C	D	pAvsB	pAvsC	pAvsD	pBvsC	pBvsD	pCvsD
Passage0	76,21	113,15	26,09	103,13	n.s	<0.05	<0.05	<0.05	n.s	<0.01
Passage1	38,33	30,17	23,52	70,34	<0.05	<0.01	n.s	n.s	n.s	<0.05
Passage2	37,34	34,76	24,43	36,23		n.s	<0.05	n.s	n.s	n.s	<0.01
Passage3	61,05	42,23	19,53	32,46	n.s	<0.01	n.s	<0.01	n.s	<0.05
Passage4	40,64	30,45	21,05	38,74	n.s	<0.05	n.s	n.s	n.s	<0.05
Passage5	48,52	45,62	25,01	41,77	n.s	<0.01	<0.05	n.s	n.s	<0.01

PO-046

GMP-EXPANDED MESENCHYMAL STROMAL CELLS: ASSESSMENT OF QUALITY CONTROLS OF IMMUNE REGULATORY PROPERTIES FOR APPLICATION IN REGENERATIVE MEDICINE (EUROPEAN MULTICENTER STUDY CASCADE).

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Introduction. Aim of the European Consortium CASCADE is to standardize GMP-grade production and clinical use of Mesenchymal Stromal Cells (MSC) to treat skin and corneal wounds. MSC possess immunogenicity and immunomodulatory properties that must be carefully addressed before clinical use. CASCADE Immunological Unit is aimed to set up and validate a wide panel of functional assays to fully characterize in a standardized and reproducible manner the immunomodulatory properties of MSC obtained inside CASCADE Units from bone marrow, adipose tissue, cord blood, and amniotic membrane (BM, AT, CB, AM) through different GMP-grade expansion protocols including platelet lysate- and fetal calf serum-based culture conditions. *Methods.* Primed MSCs (pMSCs) were obtained by 48h-treatment with rh-INF and rh-TNF. Cocultures were set up by plating MSCs or pMSCs in presence of T, B, NK cells. At 4 or 6 days of coculture proliferation was evaluated by CFDA-SE dilution. T cells were stimulated with CD3 plus CD28 antibodies; B cells were activated with CD40L, its enhancer, IL-2, CpG 2006, and anti-IgM/IgA/IgG; NK cells were activated with rhIL-2. Moreover cocultures, were performed in the presence of specific inhibitors: L-1MT (IDO inhibitor), snPP (HO-1 inhibitor), NS-398 (COX2 inhibitor), L-NMMA (iNOS inhibitor) and anti-IFN antibody. For MSCs immunogenicity assay, the proliferation of allogeneic T cells was evaluated at day 5 of culture; in addition, NK cells were activated for 2 days with rh-IL2 whereas MSCs and pMSCs were used as target cells. *Results*. Inflammatory milieu significantly upregulated MHC class I and II, CD54, CD106, CD40, CD274, CD112, CD155 expression, and downregulated NKG2D ligands (ULBP 1-3, MICA/B) and mesenchymal markers (CD73, CD90, CD105). AT-derived MSC expressed less MHC class II, CD200 and CD106 molecules than BM-MSC. MSC coculture inhibited T and NK cell proliferation without inducing apoptosis, and this effect was greater in presence of primed MSC. On the contrary, only primed MSC were capable of suppressing B cell proliferation. In addition, MSC inhibited apoptosis of resting T, B, and NK cells, while inflammatory priming increased their pro-survival activity. T cell/MSC coculture showed that activation of IDO and HO-1 was the main mechanism involved in MSC immune modulation, as the addition of specific inhibitors (L-1-MT and snPP) significantly reverted the phenomenon. MSC never promoted allogeneic T cell proliferation; by contrast, IL-2-activated NK cells could efficiently recognize and kill allogenic unprimed MSC. However, MSC became insensitive to NK cells once primed with inflammatory cytokines. *Conclusions.* Some differences were observed depending on the origin and culture conditions of clinical-grade MSC. All the experimental protocols to assess MSC inhibitory effects on immune effector cells have been standardized and will be applied for the release of GMP-grade MSC produced inside the CASCADE Consortium.

PO-047

NATURAL KILLER CELL RECOGNITION OF ACUTE LYMPHOID LEUKEMIAS

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Introduction. It is now well recognized that the activity of natural killer (NK) cells against leukemic blasts depends on the interaction between inhibitory receptors for major histocompatibility complex (MHC) class I molecules and activating receptors, which play in concert to induce the elimination of tumor cells. Among the activating receptors relevant for tumor cell recognition and killing, an important role is played by NKG2D, the receptor for the MHC I-related molecules MICA/B and ULBP1/2/3 on blast cells, and by DNAM-1 (DNAX accessory molecule-1), which recognizes the Nectin-2 (Nec-2, CD112) and the poliovirus receptors (PVR, CD155). Both NKG2D and DNAM-1 cooperate in the induction of NK cell killing of tumor cells of different histotypes, including those of hematopoietic origin. Aim of this work was to analyze the expression of ligands for these activating receptors on adult acute lymphoid leukemic (ALL) primary blasts and their functional role in NK cell recognition. *Methods.* The phenotype of primary human adult ALL blast cells was assessed by flow cytometry on a FACSCanto using anti-human monoclonal antibodies (mAbs) directed against MICA, MICB, ULBP1, ULBP2, ULBP3, Nec-2, PVR, MHC class I, CD7 and CD19. In order to evaluate the functional role played by these receptors in NK cell recognition, NK cell mediated cytotoxicity against ALL primary blasts was evaluated using the (51)Cr-release cytotoxic test in the presence or not of anti-NKG2D and anti-DNAM-1 neutralizing mAbs. *Results.* Adult ALL blast cells presented a substantial high superficial expression of ligands for the NK activating receptors NKG2D and DNAM-1 (Figure 1), thus revealing a possible mechanisms of NK cell recognition. As expected, the expression of PVR and Nec-2 on one side and of MICA and MICB on the other side were inversely correlated. B-lineage Ph+ ALL blasts, when compared to B-lineage ALL blasts carrying no known molecular markers or T-ALL blast cells, presented a higher expression of ULBP1 and ULBP3 protein receptors, confirming previous data regarding their immunogenicity. Cytotoxic assays were then performed and allowed to confirm that ex vivo-generated allogeneic NK cells mediate an efficient lysis of primary ALL blasts (>50% cytotoxicity at an E:T ratio of 50:1); interestingly, preliminary data obtained from these tests performed in the presence of anti-NKG2D and anti–DNAM-1 neutralizing mAbs indicate a possible pathway of recognition of ALL blast cells in the setting of the Nec-2/DNAM-1 interaction. *Conclusions.* These data reveal a possible pathway of interaction between NK cells and ALL blasts, and suggest the possible *in vivo* use of ex vivo generated NK cells for the control of minimal residual disease or for patients that because of age or co-morbidities may not undergo standard chemotherapeutic protocols.

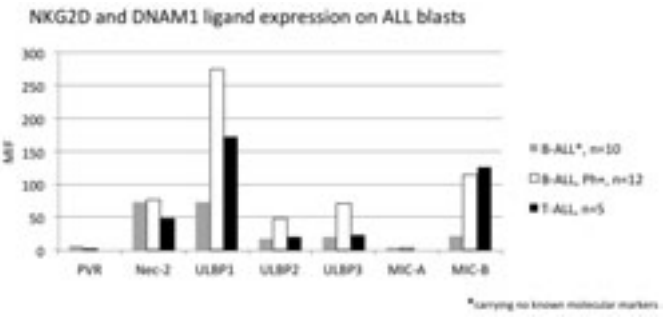

Figure 1.

PO-048

TOLL-LIKE RECEPTOR EXPRESSION AND INFECTIOUS COMPLICATIONS AFTER ALLOGENEIC STEM CELL TRANSPLANTATION

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Introduction. Despite anti-infective prophylaxis, bacterial, fungal and viral infections remain an important complication after allogeneic stem cell transplantation. Recovery from infections depends on the complete integration and balancement of innate and adaptive immune responses. In this complex interplay, Toll-like receptors (TLRs) play a key role and recognize pathogen-associated molecular patterns (PAMPs), such as common protein, carbohydrate or DNA/RNA pattern motifs. Extracellular PAMPs, especially of bacteria and fungi, are recognized by surface TLRs (TLR-1,TLR-2,TLR-4,TLR-5, and TLR-6). Intracellular TLRs (TLR-3,TLR-7,TLR-8 and TLR-9) bind mainly to foreign nucleic acids. TLRs are also receptors for endogenous ligands and damaged tissue, suggesting that both pathogen-derived molecules and products of damaged tissue can trigger signals, which may be responsible for the regulation of innate and adaptive immune responses. To our knowledge, no studies deal with expression and function of all human TLRs together in relation to infectious complications in the setting of allogeneic SCT. In this study we analyse 9 TLRs on T- lymphocytes and monocytes in relation to bacterial, fungal and viral infections (especially CMV) in the early period of allogeneic SCT. *Methods.* The expression of TLRs on T-lymphocytes and monocytes was analysed in 35 patients by flow cytometry as mean fluorescence intensity. Functional data were obtained by ELISA assay after TLRs activation. The cell supernatants were collected and assayed for TNF-alpha, IL-4, IFN-gamma, and MCP-1. Relative induction of these cytokines was calculated in relation with unstimulated controls. *Results*. Bacterial infections were associated with increased expression of TLR-5 on monocytes (P=0,04) and lower values of MCP-1 and TNF-alpha relative induction upon TLR-3 and -7 activation (P<0,05). Patients with fungal infections showed a decreased expression of TLR-7 on T-lymphocytes (P=0,02). T-lymphocytes and monocytes of patients with CMV reactivation had an increased expression of TLR-5 (P<0,05), whereas monocytes displayed lower levels of TLR-8 (P=0,03). CMV reactivation was also associated with higher values of IFN-gamma induction upon TLR-2,-3,-4,and -9 activation (P<0,05). *Conclusions.* In our study, bacterial, fungal, and CMV infections seem to be associated with a different expression of some TLRs and cytokine induction. TLR-5 and -7, which are not reported to recognize CMV and fungi, seem to be instead involved in the interaction between these pathogens and the immune system of transplanted patients. Functional analysis suggests also an atypical involvement of some other TLRs. These results could be explained by the defective immune system after SCT. The assessment of a larger number of patients, and the analysis of other possible interfering factors, could confirm our findings.

PO-049

TOLL-LIKE RECEPTORS ON PERIPHERAL BLOOD CELLS AND ACUTE GRAFT-VERSUS-HOST DISEASE AFTER ALLOGENEIC STEM CELL TRANSPLANTATION: RESULTS OF A PROSPECTIVE STUDY

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Introduction. Pattern recognition receptors such as Toll-like receptors (TLRs) play a key role in the cross-talk between innate and adaptive immune system. TLRs recognize pathogen-associated molecular patterns, such as common protein, carbohydrate or DNA/RNA pattern motifs. TLRs are also receptors for endogenous ligands and damaged tissue. Extracellular ligands are recognized by surface TLRs (TLR-1,TLR-2,TLR-4,TLR-5, and TLR-6). Intracellular TLRs (TLR-3,TLR-7,TLR-8 and TLR-9) bind mainly to foreign nucleic acids and sometimes detect self DNA/RNA. Studies dealing with some TLRs and acute graft-versus-host disease (aGVHD) report contrasting results, and there are no studies that relate aGVHD with expression and function of all human TLRs together. *Methods.* We prospectively evaluated the expression of 9 TLRs by flow cytometry on T-lymphocytes and monocytes of 34 patients in relation to aGVHD. TNF-alpha, IL-4, IFN-gamma and MCP-1 induction upon TLR activation was assessed by ELISA on cell supernatants. *Results.* Nineteen patients developed aGVHD at a median time of 28 days (range, 20-50) after transplantation. A 2-step multivariate analysis was performed using principal component analysis and multi-factor analysis of variance. The levels of TLR-5 expression on monocytes and T-lymphocytes positively correlated to aGVHD (P=0,01), whereas levels of TLR-1 and -9 resulted as negative predictors (P<0,05). *Conclusions.* This TLR profile (TLR-1, -5, and -9) could promote an overall immunostimulatory/pro-inflammatory response. A decreased expression of TLR-9 (receptor of self or non-self DNA) could promote TLR-7 activation, inducing type I interferons and other pro-inflammatory cytokines. TLR-1 and -5, which recognize components of bacterial cell wall and endogenous danger signals from injured tissues, could also modulate immune reactions in aGVHD.

PO-050

B CELL COMPARTMENT ENGRAFTMENT AFTER ALLOGENEIC STEM CELL TRANSPLANTATION AND CRONIC GRAFT VERSUS HOST DISEASE DEVELOPEMENT

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Introduction. The pathogenesis of chronic Graft versus Host Disease (cGvHD) is still unclear. Previous studies demonstrated a B cell compartment involvement, in particular with the evidence of appearance of autoantibodies after allogeneic stem cell transplantation (allo-SCT) and with the successful treatment of certain cases of cGvHD with Rituximab, a monoclonal anti-CD19 antibody. *Methods.* We investigated recovery and chimerism of B lymphocytes and circulating levels of B cell activating factor (BAFF) cytokine after allo-SCT in 82 hematological malignancy patients, median age 51 years (range 20-69), who underwent allo-SCT. We evaluated immunological recovery by flow-cytometry, hematopoietic chimerism by STR-PCR in whole peripheral blood (PB), granulocyte fraction and immunomagnetically sorted CD19+ cells, and BAFF serum levels before allo-SCT and at day 90, 120 and 180. *Results.* Forty-three patients developed cGvHD at a median time of 5 months after allo-SCT, with at least 2 organs involved and a median score of 3 according to the Organ Scoring System. In 25 cases the cGvHD progressed from a pre-existent acute GvHD. The CD19+ cell counts were similar between the two groups through the first 120 days, and both cohorts showed a deep B lymphocytopenia during the first 3 months. The median CD19 cell count at day 90 was equal to 3,8/µL, with a median of 4.7/µl in cGvDH positive patients and 1.9/µL in cGvHD negative patients. B cell levels raised at day 180 in both groups, but they increased slightly more in cGvHD negative patients then in positive ones (141/µL versus 61/µL). We could analyzed B cell chimerism at day 90 only in sporadic cases, due to the low count of CD19+ cells, but at day 120 we found full donor chimerism (FDC) on CD19+ cell fraction of all patients who had reached FDC in the whole PB and in the granulocyte fraction. BAFF kinetics was similar in the two cohorts. BAFF levels raised to a peak at day 90 in both groups (7368 pg/ml in cGvHD negative versus 7764 pg/mL in cGvHD positive) and then progressively decreased at day 180 (respectively 6157 pg/ml and 6422 pg/ml). The mean BAFF/CD19+ cell ratio presented lower values in cGvHD negative patients at 180 days (406 versus 119), parallel to the observed faster B cell recovery. *Conclusions.* We observed a severe B lymphocytopenia through the first 180 days in patients with and without cGvHD, but the B cell compartment presented a complete donor chimerism at day 120 in all patients. Moreover patients who developed cGVHD had a delayed B cell recovery and a higher BAFF/CD19+ cell ratio at day 180. In contrast, chronic GvHD negative patients seemed to reconstitute the B cell compartment faster starting from 6th month after transplantation.

PO-051

18F-FDG PET SCANNING IN GRAFT-VERSUS-HOST DISEASE AFTER ALLOGENEIC STEM CELL TRANSPLANTATION

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Background: Diagnosis and onset prediction of Graft-versus-host disease (GvHD) after allogeneic stem cell transplantation (ASCT) as well as monitoring of its treatment is still an open issue. *Aim.* To evaluate the role of positron emission tomography (PET) using the positron-emitting radionuclide 18F labeled with Fluorodeoxyglucose (18F-FDG) for the assessment of GvHD after ASCT. *Methods.* We retrospectively reviewed patients with lymphoma and multiple myeloma who underwent ASCT between 2004 and 2009 at "L. & A. Seragnoli" Department of Haematology, Bologna - Italy. 36 patients (8 HD, 20 NHL, 8 MM) has been extracted in whom a pre-transplantation PET scan and at least three post-transplantation follow-up PET scans were available. GvHD diagnosis was based on clinical data and, when available, on biopsy results. Pre-transplant and post-transplant PET scans were evaluated and compared for each patient by two experienced nuclear medicine physicians blinded by GvHD clinical status. Scans performed at the time of GvHD were compared with scans obtained before transplant and during clinical remission of GvHD, as well as with scans performed in patients without GvHD. Pathological FDG uptake in GvHD target organs was recorded. *Results.* Overall 25/36 patients developed GvHD (22 acute, 6 acute and chronic, 3 chronic) while 11 remained GvHD-free during the time of follow up. In 11/25 patients one or more PET scans were performed during the period of active clinical GvHD. In 7/11 cases pathological FDG uptake was detected in the gut (n=6), the liver (n=1) and the mouth (n=1). Six out of 11 patients had GvHD related diarrhea at the time of PET analysis. In these six cases there was intense FDG uptake in the bowel in comparison to the scans obtained in the same patients at GvHD-free periods. In 2 patients, PET analysis showed pathological FDG uptake days before the appearance of gastrointestinal symptoms. A strong decrease in bowel FDG uptake was observed in 5/6 patients responding to therapy. PET positivity was present in one of 3 cases with pathologically confirmed liver GvHD, and in one of 5 patients with oral GvHD. Among the 11 patients who did not develop GvHD, 2 patients showed a faint FDG uptake in the gut, that was also present in pre-transplant scans. Overall considering sites of documented GvHD, PET was positive in all patients with gut GvHD (n=6), in 1/3 with liver GvHD, in 1/5 with oral GvHD, and false positive in 2 patients without GvHD. *Conclusions*. Our preliminary data show a possible role of FDG-PET analysis in diagnosing and monitoring GvHD, especially of the gastrointestinal tract, when supported by clinical findings.

PO-052

DIFFERENTIATION OF MESENCHIMAL STEM CELLS (MSC) TO OSTEOGENIC TISSUE PARTIALLY REVERTS THE IMMUNOMODULATORY ACTIVITY OF THE PARENTAL CELLS ON DENDRITIC CELLS DUE TO THE DEFICIENT PRODUCTION OF IL-10, TGF- AND HLA-G

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Introduction. Preclinical and clinical studies indicate that mesenchymal stem cells (MSCs) can be used for bone repair. However, in some studies host-derived sarcomas developed around MSC implants and this was thought to be the result of the well known immunosuppressive activity exerted by MSCs, which may promote tumor development. To address this issue, we investigated whether MSC-derived osteocytes maintain the immunomodulatory capacity of the parental cells with specific reference to inhibition of dendritic cell (DCs) maturation and function. *Methods.* MSCs were isolated from human bone marrow (BM) aspirates from normal donors (hMSCs). hMSCs were cultured in osteogenic conditions. hMSC differentiated and undifferentiated were cocultured (with or without transwell) with allogeneic purified CD14+ cells in presence of GM-CSF and IL-4 and with immature dendritic cells (DCs) in presence of IL-1 , IL-6, TNF- and PGE2. hMSC differentiated and undifferentiated were cocultured with immature and mature DCs and T cells. DC differentiation and maturation, T-cell proliferation and Treg/Th17 generation were evaluated by flow cytometry. IL-10, TGF- e HLA-G secretion were evaluated in supernatants of cocultures by ELISA. *Results.* hMSC-derived osteocytes showed a lower ability to inhibit DC differentiation in comparison to their undifferentiated counterpart. Whereas the maturation and the endocytic capacity of DCs were not modulated by either MSC-derived osteocytes or undifferentiated hMSCs, the capacity of immature and mature DCs to stimulate T-cell proliferation was strongly inhibited by undifferentiated hMSCs, while was maintained by hMSC-derived osteocytes. Moreover, in contrast to undifferentiated hMSCs, hMSC-derived osteocytes partially inhibited the ability of immature and mature DCs to generate a population of CD4+CD25+ Foxp3+ T cells, which suppressed T-cell proliferation and therefore can be considered Tregs. Transwell experiments suggested the involvement of soluble factors. Quantification of the main immunosuppressive mediators (IL-10, TGF- e HLA-G) by ELISA showed that hMSC-derived osteocytes are deficient in the production of these factors in comparison to undifferentiated hMSCs. *Conclusions.* This study suggests that the differentiation of hMSCs to osteocytes partially reverts the immunomodulatory activity of the parental cells. Thus, differentiation of hMSCs to osteocytes before implantation could prevent the suppression of the host DC-mediated immune responses.

Physiopathology of Hemopoiesis

PO-053

MESENCHYMAL STEM CELLS FROM SHWACHMAN-DIAMOND SYNDROME (SDS) DISPLAY NORMAL FUNCTIONS AND DO NOT CONTRIBUTE TO HEMATOLOGICAL DEFECTS

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Introduction. SDS is a rare autosomal recessive inherited disorder characterized by bone marrow dysfunction and exocrine pancreatic insufficiency. SDS patients show an abnormal bone marrow stroma in terms of its ability to produce fat clusters and to support and maintain hematopoiesis. Recently, a role of osteoprogenitors-mesenchymal stem cells (MSCs) in inducing independent genetic mutations and frank leukaemia has been proposed. *Aims.* In this study, for the first time, we deeply characterized MSCs obtained from SDS patients (SDS-MSCs) by investigating their possible defects in supporting hematopoiesis. *Methods.* MSCs obtained from 27 SDS patients were tested for the expression of specific surface markers, their ability to differentiate into mesengenic lineages, their capability to abrogate T cell proliferation and their capacity to prevent neutrophil apoptosis. In addition, in order to asses the ability of SDS-MSCs to support and maintain the hematopoiesis, we took advantage of long-term bone marrow cultures, to mimic *in vitro* the interactions between the hematopoietic progenitors and the microenvironment of the BM stem cell niche. *Results.* MSCs derived from SDS patients (SDS-MSCs) displayed typical fibroblastoid morphology; they were consistently devoid of contaminating hematopoietic cells, but expressed common MSC markers. Similarly to MSCs obtained from healthy donors (HD-MSCs), these cells were able to differentiate into adipocytes, chondrocytes and osteoblasts. In addition, SDS-MSCs drastically decreased the mitogen-induced lymphocyte proliferation. We also cultured neutrophils obtained from HD in presence or absence of MSCs at different time points. We demonstrated that SDS-MSCs were comparable to HD-MSCs in supporting the viability of neutrophils. Importantly, SDS-MSC produced high amount of IL-6, a crucial cytokine involved in the protection of neutrophils from apoptosis. We also co-cultured SDS-MSC and CD34+cells. CD34+total number of non adherent cells, was calculated during the first 3 weeks of co-culture and were found to be similar. After an initial decline at day 7, the total number increased after 14 days of co-culture. As expected, after 21 days the percentage of CD34+ cells diminished up to 20%. Importantly, SDS-MSCs as well as HD-MSCs were able to preserve the stemness of recovered cells, as evaluated by their ability to form hematopoietic colonies in methylcellulose. *Conclusions.* Despite their pivotal role in the bone marrow niche, our data suggest that MSC themselves do not seem to be responsible for the hematological defects typical of SDS patients. Our results may facilitate future studies on biological bases underlying SDS. The identification of signals from the microenvironment could pave the way to new highly targeted strategies for the prevention an treatment of this syndrome.

PO-054

SPLANCHNIC VEIN THROMBOSIS IN AN IMMUNOCOMPETENT PATIENT WITH ACUTE CMV INFECTION: A CASE REPORT

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Background. Thrombosis associated with acute CMV infection has been reported in literature mostly in a few case reports. The incidence of thrombosis among acute CMV infection hospitalized patients is 6.4%, and the incidence of acute CMV infection among thrombosis hospitalized patients is 1.9-9.1%. In immunocompetent adults. We describe the case of an immunocompetent patient who developed a splanchnic vein thrombosis in the setting of an acute CMV infection. *Case Report.* A 38 year old female woman without previous thrombotic events and no familial history for thrombosis was hospitalized in September 2011 for asthenia and persistent fever with no palpable peripheral lymphadenopathy or rash. Her complete blood count showed hemoglobin of 13.6 g/dL, a platelet count of 146x10^9/L, and a white cell count of 6.25x10^9/L, with a relative lymphocytosis. Liver function tests revealed a mild transaminitis. Serological testing for CMV showed elevated titers of IgG and IgM and CMV DNA using real-time PCR was detectable, demonstrating an active infection; serology for Legionella pneumophila, Mycoplasma pneumoniae, Rickettsia, HBV, HCV, HIV, autoimmune disorders were negative. Abdomen ultrasound scan showed a mild hepatosplenomegaly and splenoportal ectasia. She started hydration and indometacine treatment, obtaining recovery of symptoms. CT scan performed 2 weeks later documented diffuse intrahepatic portal vein thrombosis, so oral anticoagulant therapy with warfarin was started (INR target: 2-3). Screening for thrombophilia was negative. Flow cytometry revealed absence of deficience of GPI-linked proteins. Neoplastic markers and JAK2V617F mutation analysis resulted negative. Furhtermore, no other risk factors for thrombosis were found. Of note, low D-dimer level was persistently observed. CT evidence of resolution of thrombosis was documented after 3 months of warfarin therapy. At the moment patient is still on treatment. Discussion: Deep vein thrombosis (DVT), pulmonary embolism (PE), splanchnic vein thrombosis and splenic infarction are the most prevalent thrombosis associated with acute CMV infection. While DVT/PE is more prevalent among immunocompromised patients, splanchnic vein thrombosis is more prevalent among immunocompetent patients. Certain theories suggest that CMV triggers thrombosis by enhancing platelets and leukocytes adhesion to infected endothelial cells. However, the most accepted theory indicates that CMV transiently induces production of anti-phospholipid antibodies (APLAs). In our case no risk factor was apparently associated with splanchnic thrombosis except for cytomegalovirus infection. In a different way, most immunocompetent adults with CMV-associated thrombosis have other acquired or inherited predispositions for thrombosis. Physicians should be alert for symptoms and signs of thrombosis in patients with acute CMV infection, and should test CMV serology in patients with thrombosis.

PO-055

STRUCTURE AND EVOLUTION OF A NEWLY DEVELOPED ITALIAN ARCHIVE OF PNH CLONES

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Introduction. Flow Cytometry (FCM) is considered the gold standard for PNH clone detection. In 2010, an Italian archive of FCM-detected PNH clones, named ClonePNH, (http://int.clonepnh.com/), was created on a

multi-laboratory basis. The aim was twofold: a) to provide a large list of PNH clones; b) to obtain an auto-educational effect on participating laboratories. Here, we describe the structure and evolution of the archive. *Methods.* The archive was structured as a relational database containing FCM data of PNH clones identified since 2001. We evaluated: a) the number and FCM features of PNH clones identified; b) the main Reasons for Testing (RFT) leading to FCM typing; c) FCM sensitivity (i.e. mean number of events acquired per FCM clone detection); d) evolution of the archive in terms of changes in reagent utilization during the period 2001-2012. *Results.* We collected 1524 records, including negative, positive and follow-up analyses. Eighty-one institutions were involved, but just 57 (70.37%) were active. PNH clones identified were 272. Forty-five of these (16.54%) were accompanied by a PNH2 clone with a median size of 18.5% of cells. Hemoglobinuria was the most frequent RFT bringing to FCM analysis (27% of cases), followed by aplastic anemia (AA, 13%), MDS (13%), hemolytic anemia (12%), idiopathic cytopenia (10%), bone marrow failure (5%), atypical venous thrombosis (5%), other (15%). During the archive life, the spectrum of RFT widened, going from the sole hemoglobinuria in 2001 to at least six different conditions in 2012. Average of total cells acquired increased from 13,327 in 2001 to 51,563 in 2012 (monocytes: 1,854 to 18,470; granulocytes: 21,991 to 52,195; RBC: 16,136 to 83,491). Sensitivity increase was not related to RFT, being comparable in cases with hemoglobinuria (13,327 to 70,266), MDS (18,359 to 77,352) and AA (20,928 to 81,820). FLAER was used since 2007, with an increasing % of utilization, from 4% to 60% of cases. Both CD14 (monocytes) and CD66b (granulocytes) started to be used in 2001 (100% of cases) with a decrease of CD66b in 2012 (60%). By contrast, CD24 (granulocytes) and FLAER (granulocytes and monocytes) utilization progressively increased. CD59 was the most used antigen for RBC typing. The most used gating strategies were based upon physical parameters for RBC, CD45 and/or CD33 vs side scatter for granulocytes and monocytes. *Conclusions.* This is the first multi-laboratory relational database of FCM-detected PNH clones described so far. The auto-educational goal of ClonePNH was reached, since (i) general sensitivity increased progressively, with a significant burst subsequent to the archive creation, (ii) RFT became progressively more heterogeneous and appropriate, (iii) reagent choice significantly changed, allowing us to identify the most popular FCM protocol, consisting of FLAER and CD24 for granulocytes, FLAER and CD14 for monocytes, CD59 for erythrocytes.

PO-056

CLONAL EVOLUTION IN PNH: DATA FROM THE CLONEPNH ARCHIVE

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Introduction. Three types of PNH have been described: a) florid PNH; b) PNH in the context of bone marrow failure (BMF); c) subclinical PNH, in the context of BMF. In any case, Flow Cytometry (FCM) plays a crucial role in detecting and monitoring PNH clones. In BMF syndromes, as Aplastic Anemia (AA), Unexplained Cytopenia (UC) and MDS, small PNH clones can be detected, so that high-sensitivity FCM is requested. Here, we describe the evolution of PNH clones as assessed by analyzing data from the ClonePNH Archive (http://www.clonepnh.com/). *Methods.* We analyzed data from ClonePNH to evaluate: a) how many PNH clones have been identified so far and how they are classifiable on the basis of the Reason for Testing (RFT) reported in clinical request; b) how many clones received more than two FCM determinations; c) among these, which was the initial RFT as well as the fate of the clone. *Results.* Two hundred and seventy-two PNH clones were identified since 2001 and recorded into the Archive since 2010. In 45 cases (16.54%) the main PNH clone (PNH3) was accompanied by a PNH2 component, of minor size. All 272 clones were categorized into 11 classes according to their size (see Figure): 0.1%-1% (29 clones), 1.1%-10% (50 clones), 10.1%-20% (12 clones), 20.1%-30% (15 clones), 30.1%-40% (10 clones), 40.1%-50% (14 clones), 50.1%-60% (15 clones), 60.1%-70% (11 clones), 70.1%-80% (23 clones), 80.1%-90% (28 clones), 90.1%-100% (65 clones). Of interest, most clones (N=144, 53%) showed big (90.1%-100%) or small size (0.1%-10%) while the affiliation to intermediate categories was less frequent. These findings were strictly in accordance with a study by Illingworth and coworkers (ASH Meeting 2011, Abs N. 1033). Among the 272 clones, 19 received more than 2 FCM tests. Twelve of them had a follow-up study resulting in no change in category: RFT of these clones were AA (N=4), UC (N=2), Hemoglobinuria (N=3), Atypical Thrombosis (N=1), Hemolytic Anemia (N=1) and MDS (N=1). Among the remaining 7 cases, 1 had a follow-up resulting in a decrease in category (RFT was UC), two decreased up to the extinction (both with Hemoglobinuria as RFT), while four clones increased in category (1 with AA and 3 with Hemoglobinuria as RFT). Of interest, no clone jumped from "small" (0.1 to 10%) to "big" category (90.1-100%) or vice versa, demonstrating the tendency of these two classes to remain separate and stable during the follow up. *Conclusions.* Two-hundred and seventy-two PNH clones were identified. Most of clones showed a very big (90.1%-100%) or a small (0.1%-10%) size. Nineteen clones were studied with more than 2 determinations, and most of them (12/19, 63%) had no change in category. Seven clones showed some change in category (three increased and 4 decreased), but the migration between "big" and "small" category was never observed (in both directions), suggesting that these two categories are sustained by different backgrounds and pressures.

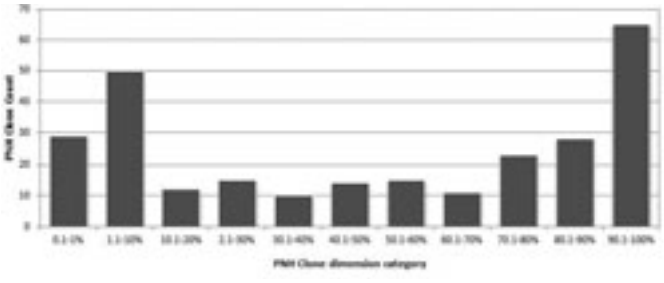

Figure 1.

PO-057

PEDIATRIC PNH CLONES: REPORT ON 16 CASES FROM CLONEPNH ARCHIVE

Cannizzo E,[1,2] Sartori M,[3] Triolo AM,[4] Lo Pardo C,[5] Buccisano F,[6], Tedone E,[7] Oliva BM,[8] Ottaviano V,[1,2] Carulli G,[1] Rosetti M,[9] De Propris MS,[10] Stacchini A,[11] Vanelli L,[12] Perbellini O,[13] Massaccesi C,[14] Gervasi F,[15] Armentano Conte C,[16] Lanza F,[17] Geuna M,[18] Del Vecchio L[19,20]

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Introduction. Paroxysmal nocturnal hemoglobinuria (PNH) is a chronic stem cell disorder characterized by deficiency of GPI-anchored proteins on hematopoietic cells. It is characterized by intravascular hemolysis variously associated with bone marrow failure and atypical thrombosis. It is a rare disease in children and little information is available. Subclinical PNH (i.e. small PNH clone in association with bone marrow failure in the absence of overt hemolysis) seems most often observed in children, particularly in the context of AA and MDS. To characterize the flow cytometry (FCM) features of PNH clone and to assess the main reasons for testing (RFT), we performed an analysis of pediatric records collected in ClonePNH Italian cytometric Archive from 2001 to 2012. *Methods.* We reviewed FCM records of 76 pediatric cases collected from 12 Regions. Sixteen cases were positive for a PNH clone. On these 16 PNH+ clones we evaluated size at first observation and during follow-up as well as the main RFT reported in the records. *Results.* The median age reported in PNH+ pediatric records was 15.5 years (1-18). Four clones were found in Campania, 2 in Emilia-Romagna, 2 in Lazio, 5 in Lombardia and 3 in Veneto. RFT included: Aplastic Anemia (AA, N=7), Hemoglobinuria (N=7), Idiopathic Cytopenia (N=1), MDS (N=1). All 16 clones were categorized into 11 classes according to sizes (see Figure): 0.1-1% = 2 clones; 1.1-10% = 2 clones; 10.1-20% = 3 clones; 20.1-30% = 1 clone; 30.1-40% = 1 clone; 40.1-50% = 1 clone; 50.1-60% = 0 clone; 60.1-70% = 1 clone; 70.1-80% = 0 clone; 80.1-90% = 2 clones; 90.1-100% = 3 clones. Of interest, most clones belonged to 80.1%-100% category (N=5) or 0.1-20% category (N=5). Just 4 clones showed a size ranging between 20.1% and 70%. These results are quite similar to those we observed on PNH in adults (N=272). Among these 16 pediatric records, 4 were sequentially analyzed with more than 2 determinations: 1 clone had a big size and the follow-up study resulted in no change of category (90.1%-100%; RFT was Hemoglobinuria); 2 clones had a small size with a follow-up resulting in no change of category (10.1%-20%; RFT was AA) and 1 clone had a small size with a follow-up resulting in a slight increase of category (from 1.1%-10% to 20.1%-30%; RFT was Idiopathic cytopenia). *Conclusions.* We observed that most of pediatric cases had a big (80.1%-100%) or a small clone (0.1%-20%). These data are similar to those observed on adults (see E. Cannizzo et al, this volume). Hemoglobinuria and AA were both the most frequent RFT where a PNH clone was detected. By contrast, in adults Hemoglobinuria was the most frequent RFT, followed by AA. Most of the PNH clones in follow-up (3/4) had no change in category, 1 with a big clone (90.1%100%; Hemoglobinuria as RFT) and 2 with a small clone (10.1%-20%; AA as RFT). These data are also similar to those observed in adults.

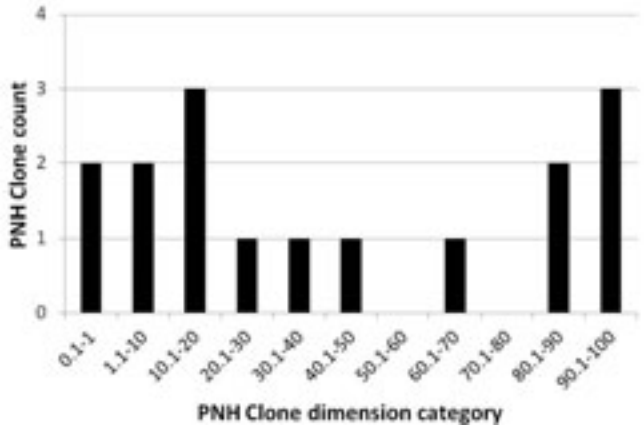

Figure 1.

PO-058

SECONDARY HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS FIVE YEARS AFTER ACUTE MYELOID LEUKEMIA : A CASE REPORT

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Hemophagocytic lymphohistiocytosis (HLH) is a systemic disorder characterized by hyperactivation of the immune system leading to proinflammatory cytokines release (IFN- , IL-2, TNF-) by T-helper cells, thus promoting blood cell phagocytosis by macrophages. There are two forms of HLH: an inherited form, named familial lymphohistiocytotis (FHL), and a secondary one (sHLH), triggered by different factors such as infections, rheumatological diseases, malignancies. Here we report the case of a 46-year-old woman diagnosed with Acute Myeloid Leukemia (AML) in March 2006. The patient was treated according to EORTC/AML12 protocol standard dose arm. After consolidation treatment, she underwent autologous stem cell transplant (SCT) and was then randomized to receive maintenance therapy with IL-2 for two years, from November 2006 to March 2008. Complete remission was maintained until August 2011, when the patient developed pancytopenia and high fever not responding to empirical antibiotic therapy. Bone marrow biopsy performed at that time was not diagnostic, only showing intense fibrosis. HLH was suspected according to biochemical and clinical manifestations, such as fever, pancytopenia, splenomegaly, increased serum ferritin concentration, hyperthriglyceridemia associated to abdominal lymphoadenopathy, generalized edema and liver failure. Pleural fluid analysis was performed, showing an hyperactivation of T –cells whereas PET-CT scan showed diffusely increased 18FDG uptake, both of them suggestive of a systemic inflammation. Diagnosis was confirmed by the presence of some figures of hemophagocytosis in a further bone marrow biopsy. The patient was treated with etoposide and dexamethasone, according to HLH-94 protocol, with an impressive reduction of serum ferritin concentration, recovery of blood cell count, reduction of spleen size and a rapid defervescence after the first week of treatment. Unfortunately, after eight weeks before continuation phase, she developed fever and increased serum ferritin levels again, and her conditions deteriorated. She underwent a second line treatment with ATG, but her conditions rapidly worsened only few days later. The patient died for disease progression and complications due to prolonged pancytopenia (sepsis, intracranial hemorrhage). This case can be considered a sHLH of unclear origin: the role of prolonged treatment with IL-2 remains obscure together with other triggering unknown factors which could have played a role in immune system dysregulation, leading to HLH. Early diagnosis of this rare disease is an essential step toward successful therapy. It should be promptly suggested by clinical and laboratoristic signs even in the absence of figures of hemophagocytosis (as they are rarely observed at disease onset). Once response to therapy is achieved, alloSCT should be performed as soon as possible, as it remains, in a little percentage of patients, the only curative treatment.

PO-059

TERAPIA COMBINATA NELLA FERROCHELAZIONE : DEFERASIROX E DEFEROXAMINA NELLA GESTIONE DEL SOVRACCARICO MARZIALE NELLE SINDROMI MIELODISPLASTICHE NEL PAZIENTE EPATOPATICO : UN UPDATE

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Myelodysplastic syndromes (MDS) are characterized by ineffective hematopoiesis, cytopenias, and a risk of transformation to acute myeloid leukemia (AML). Because the median age of the MDS onset is in the seventh decade, most patients are ineligible for potentially curative hematopoietic stem cell transplantation. Although other treatments are now available, the standard treatment for many MDS patients remains supportive care.Most MDS patients eventually become red blood cell (RBC) transfusion dependent, risking iron overload, which may lead to cardiac, hepatic, and endocrine dysfunction. Adverse effect of RBC transfusion dependence on survival was sufficiently significant that it was incorporated into the World Health Organization Prognostic Scoring System (WPSS) for MDS. Guidelines in MDS recommend chelation with an evidence of iron overload: elevated serum ferritin, iron related organ

dysfunction, or chronic RBC transfusions. Deferasirox is a well tolerated oral iron chelator drug that produces relevant benefits but, because of its potential hepatotoxicity, it is not recommended for patient with hepatic diseases. A 62-year-old man, affected by HCV positive cirrhosis and MDS (Refractory Anaemia, IPSS 0.5) started recombinant Erythropoietin therapy but it was ineffective and he underwent to a RBC transfusion program (2 blood package pro month). At a ferritin serum concentration near 700 ng/mL iron chelation therapy with deferoxamine was purposed in consideration of patient hepatic disease: compliance to subcutaneous injection was very bad, transfusion need increased exponentially until to 2 blood package pro week and serum ferritin concentration reached, in 12 months, the level of 6198 ng/mL. Since high levels of ferritin correlate with a very dangerous condition for hepatic cells, therapy with deferasirox was started but at reduced dosage (10 mg/kg/die). Before treatment start an accurate study of hepatic, renal and cardiac functions was performed. After three months serum ferritin concentration was not modified as well as other biochemical parameters, then deferasirox dosage was gradually increased reaching 30 mg/kg/die after two months and no liver damage was observed. After five months of iron chelation therapy with deferasirox at full dosage serum ferritin concentration remained very high (5098 ng/mL). Then, considering all risks related to transfusion dependent secondary hemochromatosis, with patient informed consent, a combined iron chelation therapy with deferasirox (30 mg/kg/die) and deferoxamine (2 g/day for 5 days/week) was established and after 3 months serum ferritin concentration lowered to 3000 ng/mL. In that period, Haemoglobin concentration decreased significantly, so the patient started to receive 2 RBC package pro week and, after two years of combined iron chelation therapy, serum ferritin concentration is at a stable level nearby under 3000 ng/mL. During treatment, an accurate monitoring of hepatic, renal and cardiac functions was performed: no alterations have been noted. Moreover, no serious adverse event has been observed during iron chelation therapy. The patient, after a 4-years period of transfusion therapy, died from septic shock. In conclusion, management of iron overload using combined therapy with deferasirox and deferoxamine is a safe and useful therapeutic choice in critical transfusion-dependent iron overload in MDS patients with preexisting hepatic disease.

PO-060

EXTRACELLULAR PURINES PROMOTE THE DIFFERENTIATION CAPACITY OF HUMAN BONE MARROW-DERIVED MESENCHIMAL STEM CELLS

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Introduction. Mesenchymal stem cells (MSCs) hold promise for cellular therapy and tissue repair because of their availability from a wide variety of sources and their capacity to differentiate into several cell lines. Extracellular nucleotides are potent signaling molecules mediating cell-specific biological functions. We previously demonstrated that adenosine 5'-triphosphate (ATP) inhibits the proliferation while stimulating the migration, *in vitro* and *in vivo*, of human bone marrow-derived MSC (BM-hMSC). Here, we investigated the effects of ATP on BM-hMSC differentiation capacity. *Methods.* hMSCs were isolated from BM aspirates of normal donors. BM-hMSC were transiently exposed to ATP (1mM) for 24 hours before starting differentiation induction and subsequently cultured in adipogenic or osteogenic conditions. Differentiation was evaluated by specific staining and assessment of mRNA levels of differentiation markers by qRT- PCR. Microarray analysis of ATP-treated BM-hMS differentiated cells compared with the counterpart untreated was also performed. *Results.* Molecular analysis showed that ATP treatment modulated the expression of several genes (e.g. wnt-pathway-related genes) governing osteoblastic and adipogenic differentiation of MSCs. Functional studies demonstrated that ATP stimulated adipogenic and osteogenic differentiation by significantly increasing the lipid accumulation and the expression levels of the adipogenic master gene PPAR (peroxisome proliferator activated receptor-gamma) and by promoting the mineralization and the expression of the osteoblast-related gene RUNX2 (Runt-related transcription factor 2), respectively. Furthermore, we found that while ATP stimulated adipogenesis via its triphosphate form, osteogenic differentiation was induced by the nucleoside adenosine, resulting from ATP degradation induced by CD39 and CD73 ectoenzymes expressed on BM-hMSC cell membrane. Furthermore, we used selective agonists/antagonists and gene expression analysis to reveal the contribution of specific nucleotide/nucleoside receptor activation to the stimulation of differentiation pathways. *Conclusions.* Thus, we provide new insights into the molecular regulation of MSC differentiation. We then suggest that nucleotide and nucleoside molecules may represent a novel "priming" strategy to enhance BM-hMSC differentiation for therapeutic purposes.

PO-061

COMBINED EFFECTS OF MESENCHYMAL STROMAL CELLS AND STIMULATORY CYTOKINES ON THE *IN VITRO* GROWTH OF UCB HEMATOPOIETIC PROGENITOR CELLS

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Introduction. Mesenchymal stromal cells (MSC) are a component of the hematopoietic niche that can be easily grown in vitro. Due to their ability to support expansion, MSC can be used as a primary layer of liquid cultures. The limited number of UCB HPC can result in delayed or failed engraftment in adult patients. Ex vivo expansion of HPC in co-culture with MSC may overcome this limitation. *Methods.* We have analyzed six previously frozen units of UCB. Cells were cultured in a serum free medium containing three different cytokines: granulocyte colony-stimulating factor (G-CSF), stem cell factor (SCF) and thrombopoietin (TPO), either with or without a layer of human bone marrow MSC. After 10 days we have evaluated the expansion rate of harvested cells grown with MSC both in suspension and directly adherent to the mesenchymal layer, and also of those grown without MSC. We have studied the immunophenotype of each population with a flow cytometry panel of CD45-FITC, CD34-PE, CD133-APC and 7-AAD. Furthermore the clonogenic capacity of these populations has been evaluated after a further 14 days culture in methylcellulose. *Results.* Ten days after seeding, a 2,27-fold increase (range 0,80-15,93) in CD34+ cells and a 1,04-fold increase (range 0,22-8,75) in CD34+/CD133+ cells have been obtained by cells cultured in absence of MSC. On the other hand, in cells co-cultured with MSC we have obtained a 17,14-fold increase (range 7,77-33,26) in CD34+ cells and a 4,87-fold increase (range 1,94-9,86) in CD34+/CD133+ cells. Clonogenic assay confirms our data: no immature colony forming units have been detected in the population grown without MSC, in comparison with the large number registered in the population grown with MSC. Moreover we have noticed that only co-cultured HPC generated erythroid colony forming units and their number was higher in the population of cells grown adherent to the MSC layer. *Conclusions.* Our study confirms that MSC act in a more than synergic manner on HPC expansion, so the co-culture of UCB HPC with MSC represents a good approach to overcome the problem of the limited number of UCB HPC cells. Both the infusion of expanded UCB HPC and the co-infusion of expanded UCB HPC and MSC may be used in order to improve transplant engraftment rates in adult patients.

PO-062

PREDICTED PARAMETERS OF CD 34+ HARVEST DURING EARLY-PHASE STEM CELL MOBILIZATION IN PATIENTS WITH MULTIPLE MYELOMA. SINGLE CENTER EXPERIENCE

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Introduction. Autologous stem cell transplant, after high dose chemotherapy, is a standard treatment in patients with multiple myeloma (MM). Plerixafor has been shown to allow efficacious stem cell

(CD34+) collection in heavily pre-treated patients (pts) failing previous mobilization. We have studied the kinetics of CD34+ mobilization with a twofold aim: (a) define the predictive parameters of stem cell mobilization, (b) identify the optimal schedule for Plerixafor administration "on demand". *Methods*. This retrospective study included 45 MM pts admitted to our hematological ward, undergoing CD34+ harvest during the last 7 years (58 mobilization procedures) after first line therapy and mobilization with cyclophosphamide (CY) 3-4 gr/m^2 + granulocyte colony-stimulating factor (GCSF) or GCSF alone (n=3). GCSF was given at 5 mcg/Kg/day from day +5 and 10 mcg/Kg/day from day +10 from CY until harvest. The following parameters were considered: platelets values at day +0 and CD34+/ul and CD34+% (ISHAGE protocol) at day +11 from CY. The overall CD34+ yield was calculated from all the apheresis, with the aim to collect at least $4x10^6$ CD34+/Kg. Only the first apheresis (1A) was considered for the final CD34+ yield prediction. The following categories on 1A were considered: (1) not done (CD34+ <15/l), (2) suboptimal-poor mobilizer (CD34+ 1-$2x10^6$/Kg), (3) suboptimal mobilizer (CD34+ 2-$5x10^6$/Kg), (4) optimal mobilizer (CD34+ ≥$5x10^6$/Kg). *Results.* The mean age of pts was 59,7 years(range 42-72 years). 1A was optimal in 53,5% (median CD34+ $7,1x10^6$/Kg), suboptimal in 25.9% (median CD34+ $3x10^6$/Kg), suboptimal-poor in 8.6% (CD34+ 1-$2x10^6$/Kg) and not done in 12%. All the optimal, sub-optimal and sub-optimal-poor 1A reached the target for final CD34+ yield (60,3% at 1A). All these three categories had, at 1A, CD34+/l >15. 1A CD+34% between 0,04-0,30 was correlated to suboptimal collection in 23,5%, optimal in 3,92%, while 1A CD34+% >0,31 achieved a larger number of optimal collection (60,8%) than suboptimal (13,7%). The platelets values at day 0 were below 100/ul in 2/58 procedures and successful CD34+ harvest was obtained in both. In 2/5 cases of previous failure with CY+GCSF, we obtained successful mobilization using Plerixafor+GCSF while Plerixafor+GCSF failed in 1 patient that obtained previous collection of total CD34+ $8,6x10^6$/Kg with CY+GCSF. The median number of apheresis was 2 and total median CD34+ was $13,6x^6$/Kg for optimal mobilizer, $8,6x^6$/Kg for suboptimal mobilizer and $4,45x^6$/Kg for suboptimal-poor mobilizer. 40/45 patients underwent ASCT and the recovery time for neutrophils and platelets were 12 and 14 days, respectively. *Conclusions*. Our results indicate that CD34+ <0,04% or <15/ul, at +11 days by start of CY, are cut off important to predict CD34+ harvest failures. These data could prompt the use of Plerixafor "on demand" during early phase stem cell mobilization. These results, however, should be validated in a larger cohort of patients, including lymphoma.

PO-063

ROLE OF PKC IN MURINE PROPLATELET FORMATION

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Introduction. Platelets, produced by the cytoplasmic fragmentation of bone marrow megakaryocytes (MK), are essential for primary hemostasis, to repair microvascular damages and to initiate thrombus formation. At variance with human platelets that do not express PKCepsilon, mouse platelets are PKCepsilon positive. However, we and others have demonstrated that early human MKpoiesis requires PKCepsilon, whereas the overexpression of PKCepsilon in the late phases of MK differentiation impairs platelet production. Moreover, our preliminary data indicate that generations of PKCepsilon positive platelets may be released in pathological conditions, with effects on their aggregation potential. We here used the mouse PKCepsilon positive model to better understand the role of PKCepsilon in MK maturation. *Methods.* Primary fetal liver (FL) cells were isolated from CD1 pregnant mice. Purified FL cells were cultured up to 5 days in the presence of TPO. PKCepsilon was localized by western blot and immunofluorescence microscopy during proplatelet formation, and its expression was modulated by specific shRNA. *Results.* PKCepsilon expression increases during mouse MK differentiation and is contained into pro-platelets. Forced down-regulation of PKCepsilon strongly impairs pro-platelet production. *Conclusions*. Our data show that PKCepsilon is required for proplatelet formation in the mouse. Coherently with other model systems, the most likely functional hypothesis is that PKCepsilon could be required for the completion of platelet release, as strongly suggested by its focal expression in proplatelets. Although not required for the same function in physiological human MKpoiesis, PKCepsilon might persist in human proplatelets to help in cases of increased production, with potential additional effects on platelet physiology.

PO-064

USE OF BLEEDING SEVERITY SCORE (BSS) IN VON WILLEBRAND DISEASE (VWD): QUANTITATIVE ASSESSMENT OF BLEEDING SEVERITY IN 103 PATIENTS MONITORED IN CLINICAL PRACTICE.

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Introduction. Diagnosis of VWD can be difficult, especially in the mild forms, due to the extreme clinical-laboratory heterogeneity. According to recent studies, a significant bleeding history can be evaluated by the Bleeding Severity Score (BSS), submitting to each patient a standardized bleeding questionnaire. BSS ranges from -3 to 45. In this study we attempted to evaluate quantitatively the bleeding severity in patients with VWD, identify any patterns of bleeding symptoms, estimate the associations between BSS and laboratory features and between BSS and post-diagnosis bleeding risk. *Methods.* Clinical and laboratory data were collected from VWD Ancona database. BSS was calculated for each patient according to the model developed by Tosetto at al. for MCMDS-1 VWD (JTH, 2006), using only the bleeding symptoms occurred before diagnosis. Standard statistical methods were employed to test significance of associations between BSS and other variables. *Results.* 103 subjects were studied (41 males, 63 females), 92 with VWD type 1, 2 with type 2A, 2 with type 2N and 1 with type 3; median diagnosis age was 31 (range 3-81). At diagnosis the median of VWF:Ag, VWF:RCo and FVI-II were 48% (range 5-67%), 44% (range 8-68%), 55% (range 3-68%) respectively. In adult male population (n=34) the most common symptom was post-surgical bleeding (44%), in adult female population (n=53) was menorrhagia (49%) and in pediatric population (n=16) was epistaxis (25%). In the entire population most common symptoms were post-surgical bleeding (65,5%) and mucocutaneous bleeding, especially epistaxis (33%) and haematomas (30%). A significant statistical association was found between oral bleeding and cutaneous bleeding (P=0,03); a statistical trend association was found between epistaxis and both oral bleeding (P=0,06) and cutaneous bleeding (P=0,06). The BSS ranged from -3 to 12 and the most common BSS was 0 (20%), expressed by 13 males and 8 females. On the contrary, higher BSS values were expressed more by females than by males. In pediatric population BSS ranged from 0 to 6 and the most common BSS was 0. A significant statistical association was found between BSS and both VWF:Ag (P=0,04) and FVIII (P=0,02); there was only a statistical trend association between BSS and VWF:RCo (P=0,09). During the follow-up bleeding events occurred in 30 subjects and there was no statistical association between BSS and post-diagnosis events (P>0,1). *Conclusions.* Clinic of patients with VWD is characterized by prevalence of both post-surgical bleeding events and mucocutaneous bleeding and by tendency of bleeding symptoms to associate in typical clusters. BSS allows a quantitative assessment of clinical severity, but does not seem to be helpful in bleeding risk. BSS, although designed for VWD, can actually be helpful in diagnosis of mild bleeding disorders, mostly because it forces doctors to use a systematic approach in each patient and improve communication among those who care for them.

PO-065

SAFETY AND EFFICACY OF AN EDUCATIONAL PROGRAM IN REDUCING THROMBOTIC AND INFECTIVE COMPLICATIONS OF PERIPHERALLY INSERTED CENTRAL CATHETERS (PICCS) IN HAEMATOLOGICAL PATIENTS

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Purpose. Patients with haematological disorders frequently require the insertion of medium or long-term central venous catheters (CVCs) for stem-cell transplantation, the administration of chemotherapy, or transfusion of blood products. Although peripherally inserted central catheters (PICCs) have been in use for many years, little data exist on their use in patients receiving intensive chemotherapy. *Methods*. Evidence-based interventions were implemented in our department in December 2010, and include: 1. An high level nurse education program for correct prac-

tices and prevention of catheter-associated complications. was developed for PICC nursing team;2) The use of ultrasound guide for the insertion of the tip of PICCs, thanks to a special operator training; 3) Bedside placement and confirmed PICC tip placement by chest radiography after removal of the guidewire and before the securing of the catheter; 4) Maintenance of maximum sterile barrier precautions during PICC insertion and aftercare; 5) chlorhexidine preparation, replace 10% povidone iodine for skin antisepsis; 6) adoption of PICC patient nurse archive, including the information of weekly PICC line review at our department for each patient. Here, we carried out a clinical investigation to determine the efficacy of these interventions in reducing the rate of PICC-related complications (thrombotic events, exit site infection and other complications requiring early removal of PICCs) and to compare PICCs-specific complications (CR-BSI) with a cohort population defined as each consecutive CVC non tunneled inserted over a 6-month period (May–November 2009), and before these evidence-based interventions. Results: Ninety-five PICCs were in place for a total of 7,295 PICC days (range, 1-331 days; mean, 76,7 days), and fifty-one CVCs were inserted before these interventions (range, 3-577 days; mean, 176,2 days). Sixty-six PICCs were inserted during severe thrombocytopenia (platelets < 50 x 10(9)/L), and 70 during severe neutropenia (neutrophils < 0.5 x 10(9)/L). The majority of the patients were affected by leukaemia, and PICCs were inserted to ensure adequate access throughout chemotherapy. Other mechanical complications occurred in 11 catheters, and were accidental dislodgement (4), catheter break (3), catheter inadequate (4). Compared with CVCs group, the PICCs group was associated with a lower incidence of CRBSI complication rate during neutropenia (1,05% vs 41,17%, 0,14 vs 3,67 per 1,000 CVC days) [odds ratio (OR) 0,051; relative odds reduction (ROR) 0,98]. The rate of thrombotic complications was lower in PICCs group (0,27 per 1,000 CVC days, vs 0,70 per 1,000 CVC days) [odds ratio (OR) 0,252; relative odds reduction (ROR) 0,747]. Conclusions: Ourresults indicate that a training and competence assessment program is effective in reducing the main complications PICCs-related in haematological setting.

Table 1. Catheter outcomes, by device.

	n=95 PICCs		n=51 CVCs		
	Number	Percentage (%)	Number	Percentage (%)	
Male	48	50,5%	30	58,8%	
Female	47	49,40%	21	41,2%	
Mediane Age	Range 17-82 years	49,1	Range 23-78 years	50,5	
Type of catheter:					
Silicon rubber	6	6,3%	CVC ST (short term)	35	68,63%
Polyurethane	3	3,1%	CVC LT (Long term)	16	31,37%
Polyurethane power injectable	86	90,5%			
Definite CRBSI					
	% infection	Per 1000 CVC days	range (days)	Means (days)	OR
PICCs (n.95)	1,05	0,14	1-331	76,79	0.0151
CVCs (n.51)	41,17	3,67	3-577	176,2	
Thrombotic complications					
PICCs	2,11	0,27	1-331	76,79	0,25
CVCs	7,84	0,70	3-577	176,2	

CRBSI catheter-related bloodstream infection. PICC peripherally inserted CVC. CVC central venous catheter.

PO-066

THROMBIN GENERATION AND MICROPARTICLE-ASSOCIATED PROCOAGULANT ACTIVITY AS NEW TOOLS TO EVALUATE THE HYPERCOAGULABLE STATE IN ESSENTIAL THROMBOCYTHEMIA PATIENTS

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Introduction. Essential Thrombocythemia (ET) is a myeloproliferative neoplasm at high risk of thrombo-hemorragic events. Circulating microparticles (MP) have been found altered in vascular diseases associated to an increased risk of thrombosis. Recently, high number of plasma MP has been described in ET patients. Aim of this study was to characterize the MP functional procoagulant features and to evaluate the contribution of plasma MP to the hypercoagulable state in ET patients. *Methods*. Platelet free plasma (P-FP) was isolated from 72 ET (27M/45F; 37 JAK2V617F positive) and 67 control subjects (32M/35F). MP-dependent plasma procoagulant features was characterized by two different methods: the calibrated automated thrombography (CAT) to determine the MP-associated thrombin generation (TG), and the P-PPL/1 assay (Stago R&D) to measure the MP-associated procoagulant activity (PCA). In a subgroup of 31 ET patients and 31 controls MP-associated TG and PCA were also determined in MP-free plasma (MP-FP). Hypercoagulable state was determined by measurement of plasma Tissue Factor (TF) and activated factor VIIa-antithrombin complex (FVII-AT), which reflects the degree of intravascular TF exposure and inhibition, by ELISA. Results: P-FP from ET patients generated significantly (P<0.05) higher quantity of thrombin compared to controls, as demonstrated by the shorter lag-time (20.1±1.1 vs 26±1.7 min) and time to peak (25±1.1 vs 29.8±1.6 min) and the higher peak (63.1±4.1 vs 45.7±3.1 nM) and ETP (849.3±32.8 vs 653.8±43 nM*min). Similarly, MP-associated PCA was significantly (P<0.0001) increased in plasma from ET patients compared to controls (75±1.5 vs 85±1.6 sec). No TG and very low PCA were generated by plasma from both patients and controls after MP removal. The addition of isolated MP to autologous MP-FP restored TG and PCA to the original values. TG and PCA were significantly (P<0.05) increased in JAK2V617F positive compared to negative patients. Significant correlations were found between the PCA by PPL-assay and the different parameters of TG assay [lag-time (R2= 0.414), peak (R2= -0.542), ETP (R2= -0.514)]. MP-dependent plasma hypercoagulability was associated to significantly (P<0.05) elevated FVIIa-AT complex and TF levels in ET patients compared to controls. FVIIa-AT complex and TF levels were significantly (P<0.05) increased in the JAK2V617F positive compared to negative patients. *Conclusions.* Our results show that MP-associated TG capacity, as well as PCA, are increased in PFP from ET patients, the highest values were observed in JAK-2V617F positive patients. A prolonged generation of MPs can be deleterious, as it may exert significant procoagulant and inflammatory effects favouring adverse clinical outcomes in a variety of thrombotic diseases. Our data provide evidence for a contribution of MP to the thrombophilic state of ET patients and suggest to test MP associated TG and PCA in prospective studies to predict thrombosis in these patients.

PO-067

EVALUATION OF PNH CLONES BY HIGH SENSITIVITY FLOW CYTOMETRY IN PEDIATRIC PATIENTS WITH APLASTIC ANEMIA

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Introduction. Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hematologic disorder, derived from hematopoietic stem cells with somatic PIG-A mutations. The mutated cells express defects of proteins anchored to the membrane by the glycosyl-phosphatidyl-inositol (GPI). The clinical manifestations of the PNH clone expansion are essentially: hemolytic anemia, thrombophilia and periferic cytopenia.The presence of a PNH clone is now easily assessed throught flow cytometry by studying the lack of expression of GPI-linked mole-

cules on blood cells. It has been reported an association between aplastic anemia (AA) and development of PNH clones. In addition, patients with PNH have a reduced number of hematopoietic progenitors. This suggests a possible common pathogenetic mechanism. In pediatric field, the presence of PNH clones in patients with AA was assessed only on a small case series. *Methods.* We studied 6 patients, 4 M and 2 F, with SAA (4) or AA (2) at diagnosis; 4, 7, 8, 11, 14, 15 years old respectively and 3 patients, 2 M and 1 F, with SAA (2) and AA (1),12, 17 and 30 years old respectively , after the immunosuppressive (IS) treatment, for a total of 15 observations. The search of PNH clones was evaluated in erythrocytes, neutrophils and monocytes cells by using these markers: CD59 for GR, CD24 for PMN, CD14 for monocytes and FLAER for monocytes and PMN. *Results.* The PNH clone was identified, at diagnosis, in 5 of 6 patients (83%) with the following percentages: GR CD59- 1.25%, 0.06%, 0.3%, 0.8%, 0%; FLAER on PMN 35%, 0.09%, 0.4%, 0.3%, 0.7%; FLAER on monocytes 33.5%, 0.06%, 1.3%, 14.2%, 1%. One patient after IS therapy has been undergone to other two analysis that showed an increase of the PNH clone on GR and decrease on PMN and monocytes; a second patient, however, has been evaluated by a single analysis, after IS therapy, that showed reduction of the PNH clone in PMN and monocytes. In three patients, evaluated after 20, 3 and 2 years from IS, two were positive for the PNH clone as follows: GR-CD59 5.2%, 0.02%; FLAER on PMN 35.8%, 0.26%; FLAER on monocytes 32%, 0.5%. One of these patients underwent two other additional analysis showing a substantial stability after one year. Moreover, all patients were asymptomatic for PNH. *Conclusions.* The PNH clone is present at diagnosis of SAA in a percentage greater than those previously reported and the increase appears to be associated with IS therapy or persistence of AA after IS. It is required a larger prospective study to determine the prognostic value of the PNH clone in patients with AA.

PO-068

MANAGEMENT OF SPONTANEOUS BLEEDING EPISODES AND PROPHYLAXIS IN FACTOR VII DEFICIENCY. A PROSPECTIVE EVALUATION OF 112 CASES FROM THE SEVEN TREATMENT EVALUATION REGISTRY (STER)

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Background. Patients with inherited factor VII (FVII) deficiency display different clinical phenotypes requiring ad hoc management. This study evaluated treatments for bleeding episodes and prophylaxis using data from the Seven Treatment Evaluation Registry (STER). Material and *Methods*. One-hundred and one spontaneous bleeding episodes (BE) were analysed in 75 patients (41 females; FVII coagulant activity [FVIIc] <1–20%) and 38 prophylaxis courses (P) were administered to 37 patients (22 females; FVIIc <1-5%). BE were grouped as haemarthroses (n=30), muscle/subcutaneous haematomas (n=16), epistaxis (n=12), gum bleeding (n=13), menorrhagia (n=16), central nervous system (CNS; n=9), gastrointestinal (GI; n=2), and other (n=3). P was instituted mainly for the severest symptoms (central nervous system, gastrointestinal bleeding, haemarthrosis). *Results*. Of 93 evaluable BE, 76 were treated with recombinant activated FVII (rFVIIa), eight with fresh frozen plasma (FFP), seven with plasma-derived FVII (pdFVII), and two with prothrombin-complex concentrates. Twenty-three patients received rFVIIa and 14 plasma-derived (pd) products (pd-FVII [n = 3], FFP [n = 10]) for prophylaxis. P schedules were clustered in "frequent" courses (3 times weekly), and "infrequent" courses (twice weekly or less). One-day replacement therapy for BE resulted in very favourable outcomes in haemarthroses, and was successful in muscle/subcutaneous haematomas, epistaxis and gum bleeding. For menorrhagia, single- or multiple-dose schedules led to favourable outcomes. A subset of patients with FVII deficiency needing P because of severe bleeding was identified and frequent administrations (3 times weekly) resulted the most effective, providing a rationale for life-long prophylaxis in severe FVII deficiency. Overall, no thrombosis occurred; two inhibitors were detected in two repeatedly treated patients for BE, no new inhibitors developed during P. *Conclusions*. In FVII deficiency, most bleeds can be successfully treated with a single "intermediate" dose (median: 60 µg/kg) of rFVIIa. For the most severe bleeds (CNS, GI), life long-term prophylaxis is warranted.

PO-069

SUCCESSFUL OF TREATMENT WITH ROMIPLOSTIM IN PEDIATRIC PATIENT WITH REFRACTORY PTI AND ENGRAFTMENT FAILURE AFTER ALLOGENEIC BONE MARROW TRANSPLANTATION

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Immune thrombocytopenia (ITP) is an autoimmune disorder characterized by accelerated platelet destruction as well as decreased production. The standard first line treatments for patients with ITP are corticosteroids and Intravenous immune globulin (IVIg);the splenectomy is a second line therapy. Recently two new thrombopietin (TPO) receptor agonists that stimulated platelet production, Romiplostin and Trombopag, were approved for the treatment of adults with chronic and refractory ITP already undergone to splenectomy or with contraindications to it. In the pediatric children the experience with Romiplostim is limited. Herein we report an unusual case of ITP who achieved a recovery platelet with the use of Romiplostim, after allogeneic bone marrow transplantation (BMT) and engraftment failure. A 3 years old children was diagnosed on 2004 ITP, she had received therapy with steroids and IVIg attaining a response. On June 2006 she developed myelodisplastic syndrome and she was underwent to BMT from HLA identical sibling donor.Three months after transplant engraftment failure occurred with recurrence of thrombocytopenia whereby we decided to treat her with Cyclophosphamide obtained an increase of platelets but for short time and subsequently she received periodic treatments with steroid and IVIg On January 2007 the patient developed autoimmune anaemia and thrombocytopenia and she was treated with Rituximab with a short response once again. The splenectomy not was performed because the patient had not completed the required vaccinations. On August 2011 she experienced bleeding of the gums, petechiae ecchymoses and epistaxis but at this time she was not longer responder to anything treatment even for short periods therefore we decided to treat her with Romiplostim hoping to get a recovery platelet and be able to bring the patient to splenectomy. The Romiplostim starting dose was 1 µg/Kg/week and increased until 3 µg/kg. An increase in the platelet count from 11 to 92 x10/^{9}/L was obtained after three injection and platelet continued to increase rapidly during the following week up to maximum of 303 x 10/^{9}/L; and we reduce the dose at 1 µ g/kg an keeping this dose for other six week. After 14 weeks of therapy we stopped the treatment and we observed a normal count of platelets for 25 weeks; when we noted a reduction of platelets under 150x10/^{9}/L and we started again a treatment with Romiplostim maintaining a normal value of platelets. In our experience the response to treatment to Romiplostim has been high, rapidly and lasting and notably none side effects were observed despite previously multiple therapeutic lines and the young age of patient. In conclusion we believe that the use of Romiplostim is safety and well tolerated in young refractory patients and could be considered when conventional therapies fail and it required an increase in platelets count to reach the best conditions to undergo surgery with less risk.

PO-070

SIMULTANEOUS QUANTITATIVE ANALYSIS OF ADHESION MOLECULES CD64, CD11B, CD16 AND CD62L ON NEUTROPHILS AS MARKERS OF LEUKOCYTES ACTIVATION DURING LENALIDOMIDE TREATMENT

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Introduction. Lenalidomide is a second-generation immunomodulatory drug (IMiD) therapeutically active in various hematological and solid malignancies through its immunomodulatory, anti-angiogenic, and

antiproliferative properties. Lenalidomide has been shown to exert both a direct antitumour activity (by inducing apoptosis and inhibiting proliferation of tumour cells) and immunomodulation (by inducing the activation of immune effector cells, such as T cells and natural killer cells, and inducing cytokine production). Human polymorphonuclear leukocytes (PMNs) are critical elements for initiating host innate immune defense by phagocytosis, lysis of bacteria and activation of naive B-lymphocytes. In addition, PMN play critical roles in the development of adaptive immunity and contribute significantly to inflammation. An increasing number of reports have demonstrated that neutrophils synthesize and release cytokines and chemokines during infection; for that reason neutrophils are considered a cellular hallmark of acute inflammation. Neutrophil activation occurs through a number of cell surface receptors. The main physiologic cytotoxic trigger in neutrophils is represented by the surface receptor for the Fc domain of IgG (FcR) which mediate several functions (adhesion, migration, cytokines release, phagocytosis). PMNs normally express both low-affinity receptors (FcyRII,FcyRIII). The high affinity receptor for IgG, FcyRI (CD64) is constitutively expressed on monocytes, and its expression is increased by a variety of stimuli. However, little information is available regarding the role of FcyRI upregulation on peripheral neutrophils otherwise sepsis. *Methods.* We studied different group of patients (n= 20 MM; n= 20 CLL; n= 4 AML) who had been treated at our Institution between Jul 2011 and Feb 201 along with 12 healthy donors. All the patients received a lenalidomide-based regimen and were followed up for the duration of their treatment. The value of neutrophil adherence molecules CD64, CD11b, CD16 and CD62L (L-selectin) from the whole blood were determined by flow cytometry using an Epics XL-MCL four-color flow cytometer (Beckman Coulter). Neutrophils were selected according to forward and 90° light scatter paramiters. For each sample, 10 000 events were recorded. Results were expressed as intensity fluorescence mean (IFM). Isotype controls were used in all experiments. Neutrophil function was also assessed by determining the phagocytic capacity and cytokines productions. *Results.* Lenalidomide, in all patients studied, markedly enhanced the expression of CD64 on neutrophils surface (P< 0.05). Only little difference was observed among the different hematological disease monitored. This alteration was not accompanied by an increase in CD11b, CD16, CD62L expression in neutrophils and occurred despite the progressive lenalidomide-associated neutropenia. No change was observed in monocyte counting and cell-surface markers expression. We also observed an expansion of the lymphocytes CD56+ and this data significantly correlated with CD64 up-regulation and lenalidomide dosage. *Conclusions.* Here, we demonstrate by flow cytometric analysis that lenalidomide up-regulate neutrophil surface expression of CD64. The data suggest that PMNs activation under lenalidomide modulation is indicative of enhanced innate immune functions that involved T/NK lymphocytes interactions. Further studies are need to understand how Lenalidomide directly regulates various elements of neutrophil function.

PO-071

PREDICTORS OF THE MODERATE THROMBOCYTOPENIA (MTCP)IN HIV-INFECTED PERSONS IN THE ERA OF HIGHLY EFFECTIVE ANTIRETROVIRAL THERAPY (HAART)

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Background and Aims of the study. Before the availability of HAART, TCP was a frequent finding in HIV-infected persons. Several factors contribute to low platelet (PTL) counts during HIV infection and a relationship between PTL count and disease progression has been reported. Aim of the present study was to assess the correlates of risk for moderate TCP (mTCP) since establishment of HAART in 1996. *Methods.* Predictors of mTCP (platelet count <50,000 /uL) were assessed in a cohort of adult HIV-infected patients consecutively enrolled for medical care from 1985 through 2011. Predictors were analyzed by means of Cox proportional hazards regression model including gender, age, risk factor for HIV infection, AIDS diagnosis, CD4 cell count, year of enrolment, and use and type of antiretroviral therapy as covariates. *Results.* Among the 5117 patients included in the analyses, 88% were males; mean age was 35 years (± 9.2); 23% had AIDS; 21.3% had CD4 cell count <50/uL, 33.3% between 50-249/uL, 18.9% between 250-399/uL, and 30% ≥400/uL; 69.3% had PTL count ≥50.000/uL; 43% were on antiretroviral therapy (13.4% on monotherapy, 5.3% on dual therapy, and 14% on a triple or more drug regimen); 19.6% were enrolled before 1989, 45.5% between 1990-1996, and 54.7% after 1996. mTCP was found in 706 patients (13.8%) during the follow-up. Baseline predictors of mTCP were clinical AIDS (AHR 2.3; 95% CI: 1.9-2.8), PTL counts <150.000/uL (AHR 2.3, 95% CI 1.9-2.7) and <100.000/uL (AHR 5.3, CI 4.3-6.5), and a history of injecting drug use (AHR 1.4; 95% CI: 1.1-1.7). Conversely, baseline CD4 cell count >400/uL (AHR 0.7; 95% CI: 0.5-0.9), being enrolled after 1996 (AHR 1.4; 95% CI: 1.1-1.7), and use of HAART (AHR 1.4; 95% CI: 1.1-1.7) were protective factors. *Discussion*. The risk of TCP was significantly reduced in patients enrolled after 1996 compared with those included before 1990 and in patients taking HAART. The causes of TCP observed in the HAART era deserve further investigation, although the increased risk of TCP found in drug users, a population with high prevalence of HCV co-infection, suggests also a relevant role of liver impairment.

PO-072

CANCER AS A PREDICTOR FOR INCREASED PERIPROCEDURAL BLEEDING DURING SURGERY OR PLATELET-INDUCED CHEMOTHERAPY IN CHRONICALLY ANTICOAGULATED PATIENTS

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Background. Patients with active cancer are often on chronic anticoagulation and frequently require interruption of this treatment for invasive procedures and/or chemotherapy. Few data are available on the impact of cancer on periprocedural thromboembolism (TE) and major bleeding. Patients and *Methods.* Over a period of 6 years (2003-2008) 484 consecutive patients referred for periprocedural anticoagulation (491 procedures) using a standardized protocol were followed forward in time to estimate the 3-month incidence of TE, major bleeding and survival stratified by anticoagulation indication. Active cancer and bridging heparin therapy were tested as potential predictors of TE and major bleeding. *Results.* The incidence of venous thromboembolism (VTE) complications among cancer (n. 156) and non-cancer patients (n. 328) was: in low-risk group 1.4% versus 0.54% (P= 0.02) and in high risk group 4.5% versus 3.4% (P= ns). The incidence of major bleeding was 5.5% among cancer patients undergoing major surgery and 4.4% in those with platelet-induced chemotherapy; in non-cancer patients major bleeds occurred in 2.1% of patients (P= 0.03 and P= 0.006, when compared respectively to the above cited group of cancer patients) *Conclusions.* Cancer patients anticoagulated for VTE experience higher rates of periprocedural major bleeding; the incidence of VTE was higher in the subgroup of cancer patients at low-risk for thrombosis when compared to the same population of non-cancer population.

PO-073

PREDICTORS OF RESIDUAL VEIN THROMBOSIS AFTER CANCER-RELATED DEEP VEIN THROMBOSIS OF THE LOWER LIMBS

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Background. Persistance of Residual Vein Thrombosis (RVT) after stopping anticoagulation in patients with Deep Vein Thrombosis (DVT) correlates with an increased risk for recurrent Venous Thromboembolism (VTE) in cancer and non-cancer population. Predictors of RVT in cancer patients are unknown. *Objectives.* To determine the 6-month presence and independent predictors of RVT in cancer patients treated with low-molecular weight heparin (LMWH) because of an episode of cancer-related DVT of the lower limbs. *Methods.* In a protocol driven, cohort study design, all patients referred for cancer-related DVT of the lower limbs (2006-2011; n = 382), were followed forward in time to determine the 6-month cumulative presence of RVT (Kaplan-Meier product limit) and its predictors (Cox proportional hazards). *Results.* The 6-month cumulative presence of RVT was 32.4% (124/383). RVT occurred more frequently in patients with advanced cancer. Independent predictors (hazard ratio; 95% confidence interval) of RVT included solid tumors and lymphomas

(2.8; 1.2-4.6), metastatic cancer (2.6; 1.3-5.4), use of haematopoietic growth factors (1.8; 1.1-3.2) and extensive index DVT (1.8; 1.2-2.6). *Conclusions.* Factors predisposing to RVT are primarily patient-specific. Presence of RVT suggests prolungation of LMWH until cancer is active.

PO-074

A CASE OF ACQUIRED CYCLICAL NEUTROPENIA ASSOCIATED WITH ANTINEUTROPHIL CYTOPLASMIC ANTIBODIES (ANCA) BINDING TO NEUTROPHIL ELASTASE (NE)

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Introduction. Cyclic neutropenia is a rare hereditary hematologic disorder due to a mutation of the neutrophil elastase gene (ELA2). It is characterized by cyclical drop of peripheral blood neutrophil counts (ANC of less than $0.5x10^9$/L), often associated with severe infections. While acquired idiopathic neutropenia is not uncommon, the occurrence of acquired cyclic neutropenia in adults has been reported only occasionally, sometimes in association with a proliferation of large granular lymphocytes (Dale 1988). Its pathogenesis is still uncertain. Antineutrophil cytoplasm antibodies (ANCA) have been detected in association with acquired neutropenia in various studies, many of them relating the presence of ANCA to the exposure to drugs or levamisole-tainted cocaine (Knowles 2009). Very recently ANCA have been detected also in two patients with acquired cyclic neutropenia (Rodrigues 2011), and their reactivity with an as yet unknown 60kD neutrophil antigen has been demonstrated. Here we describe a unique case of ANCA-positive acquired cyclical neutropenia occurred after exposure to cocaine, which responded to the administration of immunosuppressive therapy with prednisone.

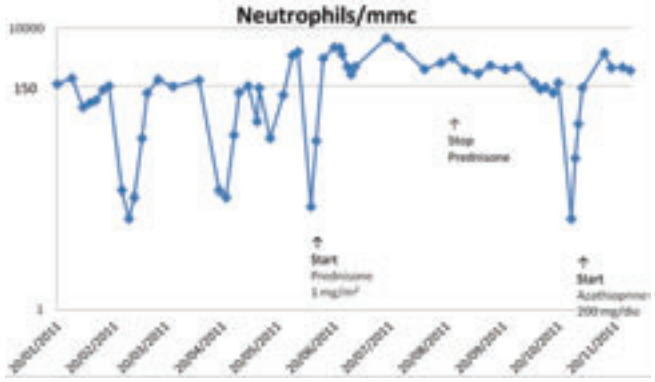

Figure 1.

Methods. Anti-MPO, anti –PR3 and anti -HNE ANCA were detected by direct immunofluorescence and anti c-myc and nickel enzyme-linked immunosorbent assays (ELISAs). *Results.* A 41-year-old woman, without familiar history of neutropenia, developed regularly recurrent episodes of agranulocytosis associated to acute tonsillitis and oral mucositis after the use of cocaine. Laboratory tests showed PR3 ANCA positivity. Clinical and hematological response to corticosteroids and G-CSF was observed at each episode. Immunosuppressive dose of prednisone was administered for 2 months, with slow tapering until stop. Neutrophil counts never dropped during the treatment. After two months the patient relapsed, and started azathioprine, obtaining remission (see figure). In order to determine how an acquired disorder could induce a cyclical breakdown of neutrophil counts, we evaluated the presence in this patient of HNE ANCA, with the hypothesis that an immune mechanism could be responsible of an accelerated apoptosis of neutrophil precursors and cyclic hematopoiesis, similarly to what happens when elastase is mutated as in congenital cyclic neutropenia. Indeed, the patient resulted positive to HNE ANCA, both by immunofluorescence assays and by anti c-myc and nickel ELISA. Evaluation of further samples from this patient and from other patients with recurrent neutropenia is ongoing to sustain our results. *Conlusions.* Our finding support a link between cyclic acquired agranulocytosis and HNE ANCA positivity, which may be induced by exposure to cocaine, and we need further studies to determine the exact pathogenetic mechanism.

PO-075

IN VITRO FERROPORTIN EXPRESSION IN NON TRANSFUSION DEPENDENT THALASSEMIA DURING ERYTHROID DIFFERENTIATION

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Introduction. Ferroportin (FPN) is the only kown iron exporter protein. It is expressed in different cell types including duodenal enterocytes, hepatocytes, erythroblast cells and reticuloendothelial macrophages. Multiple alternative transcripts of FPN with or without an iron-responsive element (IRE) in their promoter (FPN1A and FPN1B respectively) have been recently described. The expression of ferroportin and its isoforms on erythroid cells in non transfusion-dependent beta thalassemia syndromes (NTDT)is not yet fully elucidated. *Aims.* To investigate the expression profile of ferroportin isoforms during erythroid differentiation and to measure intracellular iron concentration in control and NTDT cell cultures. *Methods.* An *in vitro* model of erythropoiesis derived from human peripheral CD34+ cells from healthy volunteers and NTDT patients was used. The expression profiling of FPN isoforms (FPN1A and FPN1B) was evaluated at baseline (day 0) and at day 7 and 14 of culture by real-time PCR (2^-dCt). The intracellular iron concentration was analyzed using an Iron Assay Kit (Biovision). *Results.* In control cultures, the expression of both the isoforms FPN1A and 1B increased during erythroid differentiation, with the highest level at the end of erythroblasts stage (day 14 of cultures) (Table 1). In NTDT cultures, both isoforms expression was higher than controls at day 0 of culture (progenitors stage), noteworthy, the expression of FPN1B was significantly higher at day 0 and 14 than control cultures. The FPN1A isoform remained similar both in control and in NTDT cultures at all steps of the differentiation. The intracellular iron concentration decreased significantly during erythroid differentiation (from day 7 to day 14) both in control and NTDT cultures, however, at day 7 (early erythroblasts stage) the iron levels in NTDT cultures were notably lower than in controls. *Conclusions.* The expression of ferroportin isoforms increases during erythroid differentiation either in control than in NTDT cultures, suggesting their role in exporting the excess intracellular iron no longer needed for hemoglobin synthesis. In both conditions the FPN1A is the more expressed isoform, however is the non-iron responsive FPN1B isoform that increases significantly showing the major difference compared to control. These data suggests that FPN1B contributes to intracellular iron depletion and, consequently, to an extracellular iron overload in NTDT cultures (data not shown). The mechanisms regulating the FPN1B expression remain to be elucidated.

Table 1.

		Day 0	Day 7	Day 14
FPN1A	Control	0,15 ± 0,10	0,11 ± 0,05	3,2 ± 2,7*
	NTDT	0,33 ± 0,16	0,08 ± 0,03	3,2 ± 2,9*
FPN1B	Control	0,01 ± 0,002	0,06 ± 0,03*	0,10 ± 0,06
	NTDT	0,03 ± 0,01*	0,06 ± 0,01*	0,3 ± 0,2*°
Intracellular iron (nM)	Control	n.d.	28 ± 2,8	9,5 ± 4,5*
	NTDT	n.d.	13,5 ± 3,6*	5,8 ± 3,4*

* day 7 vs day 0: p<0.05, * day 14 vs day 7: p<0.05, ° NTDT vs control: p<0.05

PO-076

PERIFOSINE ALTERS THE SURVIVAL AND THE FUCTION OF MONOCYTES DURING DIFFERENTIATION TO APC.

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Background. APC are essential to induce immune responses, such as graft versus host disease (GVHD). Monocytes have been shown to differentiate to DC as well as macrophages *in vitro* and are considered the major source of APC *in vivo*. Perifosine is a synthetic novel alkylphospholipid antitumor agent which inhibits Akt activation and induces apoptosis of neoplastic cells, such as multiple myeloma cells. In this study we tested the effects of perifosine on the differentiation of monocytes to distinct APC subtypes *in vitro*. *Methods.* Purified monocytes from healthy donors were

cultured *in vitro* with GM-CSF (50 ng/ml) with or without IL-4 (800 U/ml) to induce their differentiation to macrophages and DC, respectively. Perifosine was added at the start of culture at the dose of 1 up to 5 uM, at a concentration lower than that used in previous work done on multiple myeloma cells. After 3 and 6 days cells were harvested and checked for their (1), apoptosis (2) expression of costimulatory and DC-differentiation molecules, (3) production of TNF- and IL-12. *Results.* Treatment with perifosine was associated with increased apoptosis of monocytes cultured both with GM-CSF only or with GM-CSF and IL-4. This effect was observed already on day 3 and starting from a concentration of 2,5 uM and appeared to be time and dose dependent. The cells cultured with perifosine showed an altered phenotype too both on day 6 and day 8 after maturation in the presence of LPS. Moreover, perifosine blocked the secretion of IL-12 and TNF- by monocytes cultured with GM-CSF only (about 50% for both cytokine; n° exP=3) and with GM-CSF and IL-4 (about 40% for both cytokines; n° exP=3) after 3 days of culture. *Conclusions*. These results suggest that perifosine acts on the survival, the phenotype and the function of monocytes during differentiation to APC independently of the presence or absence of different growth factors in the culture medium. Further studies are ongoing to evaluate the molecular mechanisms of the effect of perifosine on distinct APC subpopulations.

PO-077

FREQUENCY AND DETERMINANTS OF THROMBOTIC AND BLEEDING COMPLICATIONS IN PH NEGATIVE MYELOPROLIFERATIVE NEOPLASMS (MPN): RESULTS OF A PILOT STUDY ON 76 PATIENTS

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Background. Patients with Ph negative Myeloproliferative Neoplasms (MPN) such as Polycythemia Vera (PV), Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF) can show thrombotic and bleeding complications, with increased morbidity and mortality. Age, previous history of thrombosis, increased White Blood Cell (WBC) and JAK2 allele burden have been proposed as risk factors for Venous (VTE) and Arterial (ATE) thromboses while bleeding has been associated with abnormalities of the von Willebrand factor (VWF). *Aims.* to investigate the frequency and determinants of thrombotic and bleeding complications in a pilot study on well characterized MPN patients. Patients and Methods: 76 consecutive MPN cases were diagnosed according to WHO criteria with the following mean age, (range), gender M/F and Jak2 positivity (%): PV[n=38, 68 (36-86), 17/21; 84.2%]; ET [n=28, 66 (34-93), 9/19, 64.2%]; PMF [n=10, 70, (37-88), 7/3, 50%]. Thrombotic and bleeding episodes were recorded and managed from the time of diagnosis and associated with aspirin (ASA) intake. Among additional lab parameters VWF activities such as ristocetin cofactor (VWF:RCo) and antigen (VWF:Ag) were also measured in patient's plasma as endothelial/platelet marker. Statistics were performed by SPSS-17.2. *Results*. 56/76 (73.7%) patients with 67.8% JAK2 pos did not show any thrombotic or bleeding complications during the 6-year follow-up. In these cases mean and range values of VWF:RCo and VWF:Ag were 112, 52-202 and 140,58-288 U/dL. 14/76 (18.4%) cases with 67.8% JAK2 pos showed at least one thrombotic event (11ATE/3VTE): AMI (5), STROKE (5), TIA (2), PE (1), DVT (3). Patients with ATE showed relatively higher mean values (range) VWF:RCo=124, (61-237) U/dL and VWF:Ag=148, (84-256) U/dL but only older age (P=0.05), higher WBC (P=0.041) and higher mean platelet volume (P=0.001) were statistically significant. On multivariate analysis, age (P=0.021) and reticulated platelets (P=0.023) remained different. ATE resulted associated also with the absence of ASA intake (P=0001). Major bleeding episodes mainly mucosal (3 gastrointestinal, 3 post-surgery, 1 severe menorrhagia) requiring blood transfusions or hysterectomy were observed in 6/76 (7.9%) in cases with relatively lower mean values of VWF:RCo=77 (25-118)and VWF:Ag=106 (35-166) U/dL. At the multivariate analysis, major bleeding were significantly associated to VWF:RCo <30 U/dL(P=0.022) and/or with VWF:RCo/Ag ratio <0.6: these VWF abnormalities were also associated with ASA intake (P=0.004). Conclusions: Based on these preliminary observations, we confirm that thrombotic events have multifactorial risk factors: VWF activities might play a role as additional risk factors in ATE. Conversely, lower levels of VWF are indeed important risk factors for bleeding complications, in MPN especially in patients treated with ASA.

Lymphomas

PO-078

GENOMIC IMBALANCES CLUSTER SIGNIFICANTLY IN HIGH GRADE HCV-RELATED LYMPHOMAS

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Introduction. Low grade lymphoma regression under antiviral treatment suggests HCV plays a role in NHL pathogenesis. HCV supports mono- oligo- clonal B-lymphoproliferation, thus favouring selection of malignant clones. As genetic damage accumulates, low grade lymphoma may evolve to high-grade malignancy which, in some cases, may be present from onset as an aggressive B lymphoma. Genetic studies on HCV-lymphomas are scarce. Using FISH and Comparative Genomic Hybridization (CGH) in HCV positive patients we found a prevalence of trisomy 3q in low grade lymphomas and of 2q deletion in diffuse large B cell lymphoma (Matteucci et al Leukemia 2008;22:219-222). In the present study CGH investigated genetic imbalances in various types of HCV-related malignancies. *Methods.* The archived series of 18 patients included 5 marginal zone lymphoma (MZL), 7 diffuse large B-cell lymphoma (DLBCL), 3 follicular lymphoma (FL), 2 Hodgkin's disease (HD), and 1 multiple myeloma (MM). In all cases HCV infection was serologically demonstrated. DNA was obtained from paraffin embedded sections of pathological lymph nodes in all cases except patient n.12 whom DNA was obtained from peripheral blood. CGH sought for genomic imbalances in these DNA samples. *Results.* A total of 72 imbalances (39 gains, 33 losses) were found in 12/18 patients. The most frequent gains were found at chromosomes 7 (5 times) and 3 (4 times). The most frequent losses were found at chromosomes 10 (4 times), 2, 6, 8, 9 and 17 (all 3 times). Most imbalances (30 gains, 17 losses) were found in high grade DLBCL, as expected with only 1/7 being normal. Overall 14 imbalances were detected in the case of MM and 9 (4 gains, 5 losses) in 3 cases of FL; 2 losses were detected in 5 cases of MZL while no abnormalities in the 2 samples of HD. In high-grade lymphoma genomic complexity was significantly higher (P<0.029, Mann-Whitney test in 8 low grade HCV lymphomas versus 7 high grade HCV lymphomas). *Conclusions.* The significantly different genetic backgrounds of low- and high- grade lymphomas, suggests that information from a longitudinal series is needed to identify specific events in tumor progression. The present study confirms that loss at 2q22.3 is probably one of these steps as it was specifically associated with HCV-related DLCL (cases n.11 and n.12). It also shows that loss at 10q appears to be an early event in low grade lymphoma as it was detected as an isolated change in 2/4 cases, namely 1 MZL and 1 FL. *Acknowledgments*. FCRP: 2012.0108.021

PO-079

THE EPIGENETIC ROLE OF DAPK1 PROMOTER METHYLATION IN FOLLICULAR LYMPHOMA AS PREDICTIVE PARAMETER OF DISEASE OUTCOME: A QUANTITATIVE ASSESSEMENT BY METHYLIGHT-PCR

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Introduction. Transcriptional silencing of tumor suppressor genes, due to aberrant promoters' methylation, is a common epigenetic event in B-cell Lymphomas, including FL. Using a qualitative analysis, we previously reported that promoter hypermethylation of the pro-apoptotic death-associated kinase-1 (DAPK1) is a frequent epigenetic alteration in FL and correlates with an unfavorable outcome. The aim of our study was to assess whether a quantitative evaluation of DAPK1 methylation levels could provide additional and more accurate prognostic informations. *Methods*. We retrospectively studied 107 patients (49 males and 58 females), diagnosed at our Institution for FL from 2001 to 2011, 91 of whom were treated with immunochemotherapy (63 R-CHOP, 8 R-CVP, 11 R-FM, 9 other). DAPK1 promoter methylation, was determined on BM specimens by Methylight-PCR. As controls we included BM specimens obtained from 10 ITP patients and PB samples from 10 healthy volunteers. Quantitative results are expressed as percentage of methyla-

tion and the lower limit of detection of our assay was 0,1%. *Results.* We found DAPK1 promoter methylation in 73,8% of bone marrow samples of FL patients at diagnosis, while no methylation was found in our control group. The quantitative analysis demonstrated significantly higher methylation levels in patients with advanced disease indicated by: bone marrow infiltration (P=0.0003), involvement of more than five lymphonode sites (P<0,0001) and stage IV disease (P=0.0003). Methylation levels were lower in patients with low risk disease according to both FLIPI and FLIPI2 score (FLIPI 0-1 vs other: median 0,23% vs 0,53%; P=0,0014; FLIPI2 0 vs other: median 0,25% vs 1,12%; P=0,025). DAPK1 methylation levels were also associated to low histological grade (G1-G2 vs G3; median 0,41% vs 0,17%; P=0,004). Interestingly, patients not reaching CR after immunochemotherapy showed significantly higher levels of methylation (P=0,012), suggesting a prognostic role for DAPK1 as epigenetic biomarker. In 49 patients we studied methylation levels of DAPK-1 before therapy and at the end of treatment. All un-methylated patients before therapy (n=14), showed no methylation also at the end of treatment, while 11/35 (31.4%) of methylated ones, retained detectable levels of DAPK1 methylation. Using the 75° percentile of methylation levels (1,64%) as cutoff we found that levels higher than 1,64% predicted a significantly poorer outcome in terms of PFS (HR 3.62; 95% CI, 1,47-8.91; P=0,0051, Figure 1; analysis adjusted for treatment regimen). Including the FLIPI score into a multivariate analysis high levels of DAPK1 methylation (>1,64%) retained their impact on PFS as independent risk factor (HR: 2,8; 95% CI, 1,1-7,3; P=0,03). *Conclusions.* Our study indicates that the quantitative analysis of DAPK1 promoter methylation is a promising biomarker for Follicular Lymphoma, with prognostic impact on patients' outcome.

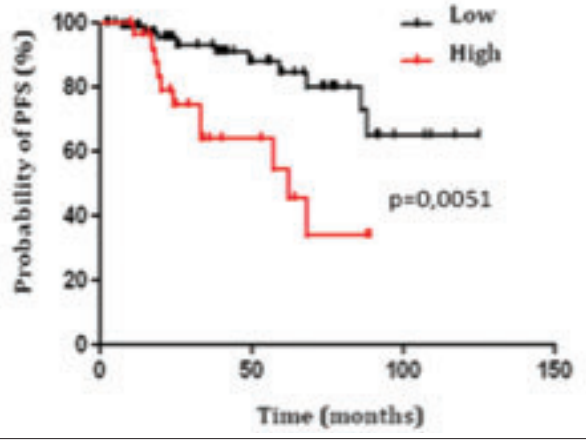

Figure 1.

PO-080

NVP-BEZ235 ALONE AND IN COMBINATION IN MANTLE CELL LYMPHOMA: AN EFFECTIVE THERAPEUTIC STRATEGY

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Introduction. Although therapies for mantle cell lymphoma (MCL) significantly improved over the last few years, however clinical course of disease is still characterized by relapses and eventually patients died for disease progression. Thus new agents are needed to improve survival outcomes. BEZ235 is a imidazoquinolines that inhibits the PI3K/Akt/mTOR signalling axis at the level of both PI3K and mTOR. In this study, we analysed the inhibitory effects of BEZ235 on MCL lines and its effects in combination with enzastaurin, everolimus and perifosine. *Methods.* Granta and Jeko cell lines were treated with different concentrations of BEZ235 (Novartis) and the IC50 value was evaluated using MTT assay. Cell viability was assessed by Tripan-blue and cell cytotoxicity with MTT-assay. Apoptosis was evaluated by Annexin V/Propidium Iodide (PI) staining and flow cytometry analysis. Cell cycle was analyzed by flow cytometry using PI and BrdU incorporation. Western blotting experiments were performed to determine if drug combinations affected PI3K/AKT, PKC and MAPK/ERK pathways. The effects of BEZ235 on bone marrow stromal cells seeded with MCL cell lines were evaluated using the MTT assay. The interaction between BEZ235 and enzastaurin, perifosine and everolimus was examined by isobologram analysis (Chou–Talalay method). *Results.* BEZ235 had the following effects: 1.significantly decreased the % of viable cells from patients with MCL but had minimal effects on PBMCs from healthy donors 2. induced significant increase (P<0.001) of apoptosis, both via intrinsic and extrinsic pathways as demonstrated by caspase 9, caspase 8, caspase 3 and PARP cleavage 3. induced an up-regulation of pro-apoptotic Bim and p-BADser112 proteins and no change in BCL-2 expression 4. induced down regulation of Cyclin D1 protein and a significant (P<0.05) decrease in the S-Phase entry of the cell cycle 5. showed a cytotoxic effect on cell lines even when cultured with BMSCs 6. had minimal cytotoxic effects on BMSCs 6. reduced expression of p-PI3k, p-Akt, p-GSK3 mTOR, p70S6 and 4EBP1, while did not affect the phosphorilation status of p-MAPK and p-P90RSK 7. enzastaurin, everolimus and perifosine enhanced the cytotoxicity triggered by BEZ235; indeed a clear synergistic interaction (CI<1) appeared after 48 hours using low concentrations of all compounds 8. decreased Bcl-2 expression in combination with enzastaurin 9. did not affect phosphorilation status of MAPK. *Conclusion.* Our study underline that BEZ235 alone effectively inhibited the growth of MCL cell lines inducing apoptosis and G1 arrest, but also we demonstrated a strong synergistic activity of BEZ235 with enzastaurin, everolimus and perifosine. In particular, the combination of BEZ235 with enzastaurin was more effective on Bcl-2 expression that either agent alone.The above results encourage clinical development of BEZ235 alone or in combination with synergistic compounds.

PO-081

RITUXIMAB-TREATED B LYMPHOCYTES AND NON-HODGKIN LYMPHOMA CELLS FEATURE A DISTINCT CLUSTERIN EXPRESSION PROFILE

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Introduction. Non-Hodgkin Lymphoma (NHL) first line of therapy consists of R-CHOP regimen, which combines chemotherapeutic drugs with Rituximab. Rituximab targets the CD20 surface antigen expressed on both normal and malignant B cells and the *in vivo* effects include antibody- and complement-dependent cytotoxicity, growth inhibition and apoptosis. Rituximab inhibits the expression of the anti-apoptotic proteins Bcl-2/Bcl-XL, down-modulating different survival pathways. Clusterin (CLU) is an ubiquitary protein involved in many physiological processes like lipid metabolism and tissue remodeling. Cancer is among the several pathological conditions in which CLU plays a relevant role even though very limited information is available up to now about its function in lymphomas. *Methods.* A diffuse large B cell lymphoma cell line (Toledo) was chosen as experimental model of NHL. Human B lymphocytes were purified from peripheral blood through negative selection (Stemcell; 4 healthy donors). Rituximab (Roche) and doxorubicin (Sigma-Aldrich) were used to treat Toledo and B lymphocytes. Phenotype analysis was performed through flow cytometry; cell viability was assessed through Trypan blue exclusion; apoptosis was evaluated by Annexin V/propidium iodide staining and flow cytometry. CLU, Bcl-2 and Bcl-6 protein levels were assessed by western blot. *Results.* Rituximab treatment (up to 100 micrograms/mL) doesn't significantly inhibit Toledo growth and does not induce apoptosis despite the fact that these cells are CD19+/CD20+. On the contrary, doxorubicin 1 microM determines a growth inhibition higher than 80% and induces necrosis and apoptosis. Consistently, Bcl-2 protein levels do not decrease after Rituximab treatment. Bcl-6 is not expressed by these cells in any of the studied conditions. CLU is expressed at very low levels by Toledo cells and seems not to be affected by Rituximab. On the contrary, doxorubicin determines a marked and reproducible up-regulation of the 60 kDa isoform. In order to have a comparison with normal lymphocytes, we purified primary B lymphocytes from peripheral blood of healthy donors. The enriched population was treated with Rituximab 10 micrograms/ml, 100 micrograms/mL and doxorubicin 1 microM. Rituximab 10 micrograms/mL and 100 micrograms/ml inhibits B cell survival of 32.7% and 41.7% respectively. Interestingly, 60 kDa CLU is higher compared to the Toledo cells and shows a different pattern in that a 55 kDa band appears only after Rituximab treatment and not after doxorubicin. This

55 kDa band is absent also in the untreated B lymphocytes. *Conclusions.* Toledo cell line is resistant to Rituximab treatment in the tested conditions and is characterized by a very low CLU protein level. CLU is not affected by the treatment. Primary B lymphocytes, on the contrary, display higher CLU levels and an additional 55 kDa band becomes evident only after Rituximab treatment but not after doxorubicin. This parallels the observed Rituximab-mediated inhibition. These results suggest a role for CLU during Rituximab response.

PO-082

IN VITRO COMBINATION OF BORTEZOMIB WITH ENZASTAURIN OR LENALIDOMIDE ENHANCES THE CYTOTOXICITY IN B-CELL LYMPHOMA CELL LINES

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Background. The therapy of patients with indolent B – cell lymphomas (B-NHL) has seen significant improvements over the last decade, especially since the introduction of monoclonal antibodies and other biological therapies in the clinic. However, clinical course of disease is still characterized by relapses and new agents are needed to improve the outcome of B-NHL. Early clinical studies of Bortezomib-based combinations, showed encouraging results both in follicular (FL) as well as in mantle cell lymphomas (MCL). In this study we hypothesize that combining Bortezomib with Enzastaurin or Lenalidomide would target separate signaling pathways increasing tumor-cell death. *Methods.* Bortezomib, Lenalidomide and Enzastaurin alone and their combinations were tested in WSU-NHL, RL (FL cell lines) and Granta-519 and Jeko-1 (MCL cell lines). B-NHL cell lines were treated for 24-48 hours. Cell viability was assessed by Tripan-blue and cell cytotoxicity with MTT-assay. Apoptosis was evaluated by Annexin V/Propidium Iodide (PI) staining. Cell cycle was analyzed using PI by flow cytometry. Western blotting experiments were performed to determine whether the drugs combinations affected PI3K/AKT, PKC and MAPK/ERK pathways. *Results.* In the present study we have shown that Enzastaurin and Lenalidomide enhanced the cytotoxicity of Bortezomib in all B-NHL cell lines and primary cells from lymphoma patients. A clear synergistic interaction, confirmed by the Chou-Talalay method (combination index<1) was observed after 24 hours using low concentrations of all the drugs. The combination of Bortezomib with both Enzastaurin or Lenalidomide did not trigger relevant decrease in the viability of normal peripheral blood mononuclear cells (PBMNCs) and suppressed cell proliferation of B-NHL cell lines when co-cultured with bone marrow stromal cells (BMSCs) in a system that mimics the bone marrow microenvironment. In comparison with each single agents, the combination of Bortezomib with both Enzastaurin and Lenalidomide induced significant increase of apoptosis, mediated by the cleavage of caspases 3 and 9. After 24 h of treatment, the combinations modified BCL-2 levels, decreased BAD phosphorylation and increased BIM expression. In the FL cell line WSU-NHL cell cycle analysis showed that the combination of Bortezomib with both Enzastaurin or Lenalidomide reduced the proportion of cells in the G0/G1, S and G2/M phase. Western blot analysis showed that anti-proliferative events were associated with dephosphorylation of PI3K/AKT, PKC and MAPK/ERK pathways. *Conclusion.* These results indicate that the combination of Bortezomib with both Enzastaurin and Lenalidomide induces synergistic anti-proliferative and pro-apoptotic effects in both B-cell lymphoma cell lines and in primary cells. This direct cytotoxicity is mediated by signaling events involving AKT, PKC, PKC and Bcl-2 pathways leading to cell death. These *in vitro* studies provide the rationale to test combinations of these active agents in patients with follicular lymphoma and mantle cell lymphoma.

PO-083

APLICATION OF TELECARDIOLOGY IN HEMATOLOGICAL PATIENT

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Telecardiology is the branch of telemedicine that through the use of ICT technologies, enables remote monitoring of health status of patients suffering from heart disease. The telecardiology is to collect vital signs, electrocardiogram and echocardiography execution, with Bluetooth wireless technology in medical devices, which send data to a touch-screen workstation connected directly to a database accessible to the cardiologist on duty. This allows to reduce the time of reporting, to have a recording of the course of the patient for the time monitoring and to reduce the discomfort related to exhausting hours in the waiting room.

In our department, we introduce the use of this technology to monitor the patient candidate for bone marrow transplantation, before and after transplantation to prevent or reduce the risk of cardiotoxicity in patients receiving anthracycline chemotherapy cycle. The prospective observational study, currently only designed for patients with anthracycline chemotherapy cycle, was sent to the ethics committee of our company to be confirmed for the beginning of the same, and includes an enrollment of 284 patients with follow-up evaluations up to 10 years. The primary objective: assess levels of acute and late cardiac toxicity in patients undergoing chemotherapy including anthracycline. General objectives:Improving the quality of life of patients;Standardize the behaviors relating to the evaluation and management of cardiovascular risk;Increase the timeliness of intervention between implementation and reporting on the electrocardiogram, echocardiography and vital signs; Tracing the evolution of the patient's clinical; Evaluate the change in echocardiographic values. Reference Documents a. Get: Medical records, the database telcardiology; b. Generated: Cardiological evaluation forms, of 'infusion of anthracyclines Cline, evaluation form of transplant patients, informed consent.

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PO-084

PROGNOSTIC IMPACT OF CONCORDANT AND DISCORDANT BONE MARROW INVOLVEMENT IN PATIENTS WITH DIFFUSE LARGE B-CELL LYMPHOMAS TREATED WITH R-CHOP

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Background. The improvement in treatment outcomes of diffuse large B-cell lymphoma (DLBCL) observed following the introduction of Rituximab has altered previous views about risk assessment. Approximately 10% to 25% of patients (pts) with DLBCL exhibit bone marrow involvement with lymphoma at the time of diagnosis. Concordant bone marrow involvement has generally been associated with a poorer outcome but the impact of discordant involvement remains less clear. The aim of this study was to examine the prognostic impact of (BM) involvement on response rate (RR), overall survival (OS) and progression-free survival (PFS) in 210 patients with *de novo* DLBCL. *Patients and methods*. Between 2000 and 2011 we retrospectively analyzed 210 untreated DLBCL pts, newly diagnosed at our centre, with or without (BM) involvement. The median age was 62 yrs (16-89 yrs), 128 (61%) were males, 90 (43%) had B symptoms, 30 (14%) had extranodal dissemination >1, 61 (29%) had a high IPI score. The treatment protocol for front-line therapy included rituximab plus cyclophosphamide, doxorubicin, vincristine and prednisone (R-CHOP). Progression-free (PFS) and overall survival (OS) were calculated according to the absence or presence of (BM) involvement and the presence of histological discordance or concordance between the primary site and the bone marrow. *Results.* A total of 48 (23%) of 210 pts had bone marrow involvement, concordant in 19 (9%) and discordant in 29 (13%). Median follow-up was 30 months (range, 1 to 142). The 48 pts with bone marrow involvement had a poorer prognosis compared with the group without (3 year progression-free survival, 55% vs 83% - P<0.05).) PFS was inferior in those with concordant (P<0.001) and discordant (P<0.05) involvement, while OS was inferior only in those with concordant involvement (P<0.001). Concordant involvement was associated with elevated lactate dehydrogenase, extra-

nodal dissemination>1, IPI>2, performance status >2 and elevated beta 2 microglobulins. Complete remission (CR) rates were inferior in concordant bone marrow involvement as compared to discordant or no involvement (45% vs 59% vs 74%). *Conclusion.* Our data show that concordant, but not discordant bone marrow involvement, could represent a possible predictive factor of poor prognosis, which would help to identify a high risk subgroup of newly diagnosed DLBCL. To confirm these results, further large-scale and prospective studies will be required.

PO-085

PROGNOSTIC ROLE OF CIRCULATING DNA IN PLASMA OF HODGKIN LYMPHOMAS

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Introduction. Cell-free DNA (cfDNA) circulating in plasma released from both neoplastic cells and the inflammatory microenvironment has been shown to be significantly higher in cancer patients and to decrease progressively during cancer remission. We have previously reported that plasma DNA levels were higher in patients with lymphomas compared to controls and significantly associated to patient characteristics and prognosis (Hohaus *et al*., Ann Oncol 2009; 20:1408). In this study, we evaluated cfDNA levels in a large group of HL patients at diagnosis and during the follow-up. cfDNA was compared with those of a population of healthy individuals and correlated to clinical characteristics and prognosis and to other biological parameters, as cytokine levels and tumor-associated macrophages. Methods cfDNA levels have been studied in pre-treatment plasma samples of 136 consecutive HL patients (62 males and 74 females) diagnosed at our Institution between 2004 and 2011, and 63 healthy volunteers (28 males and 35 females). 50 patients were also evaluated after two cycles of chemotherapy. Quantitative analysis of cfDNA has been assessed by real-time PCR for the beta-globin gene (GenBank accession number U01317) and results are expressed as ng/ml. The coefficients of variability intra- and inter-assay were both < 0.01. Results The median plasma levels of cfDNA for HL patients measured at diagnosis were significantly higher compared to controls (median 23.56 vs 12.28 ng/ml; P<0.001) and associated to advanced stage disease (stage IIB-IV vs I-IIa, P=0.03), presence of B-symptoms (P<0.001), increased LDH levels (P<0.001), and an IPS score >2 (P=0.002). Moreover, levels of cfDNA correlated with plasma levels of inflammatory cytokines as IL-6 (beta=0.35, P<0.001), and IL-10 (available in 68 patients; P=0.02) and there was a trend for higher cfDNA levels in patients with infiltration of tumor-associated macrophages (CD68+ cells >5%, available in 84 patients, P=0.06). Using the 95% upper level of controls as cut-point (>29.24 ng/mL), patients with elevated cfDNA levels had an inferior event-free survival (EFS) (P=0.0001) and an inferior overall survival (OS) (P=0.009). In a multivariate analysis including advanced stage and adjusted for the type of chemotherapy regimen, elevated levels of cfDNA remained a prognostic factor for both EFS (HR 8.2; C.I. 1.8-37.9; P=0.007) and OS (HR 6.3; 95% C.I. 1.3-33.3; P=0.03). In 50 patients, plasma was available after 2 cycles of chemotherapy and was analyzed for cfDNA plasma concentration. cfDNA levels significantly decreased in patients with elevated pre-treatment cfDNA levels, while cfDNA levels remained unchanged in patients with cfDNA levels in the normal range at diagnosis (ratio 0.33 and 1.11, respectively, P=0.003). *Conclusions.* The quantitative evaluation of plasma cell-free DNA provides a potential non-invasive biomarker for Hodgkin lymphoma, with a significant prognostic impact on patients outcome.

PO-086

COMPLETE REMISSION OF NON HODGKIN'S LYMPHOMA (NHL) AND GASTROINTESTINAL STROMAL TUMOR (GIST) WITH IMATINIB AND RITUXIMAB: AN EXTRAORDINARY CASE

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Introduction. GIST presenting synchronously or following NHL has only infrequently been reported. We report an unusually rare case of synchronous LNH lymphoma and GIST of the rectum in elderly patient treated with success with imatinib and rituximab, without chemotherapy. *Case report.* We describe a case of 85-year-old women presenting with fatigue, lymphadenopathy and weight loss. Physical examination revealed a palpable lymph node of axillas and cervical sites. Excisional biopsy of the cervical lymph node and bone marrow examination showed NHL, marginal type. Staging by CT scanning revealed lymph nodes, splenomegaly and unexpected mass in the rectum of 5.5 cm. Rectoscopy and pathological evaluation showed a GIST with CD 34 + and CD 117+ . The bone marrow was involved and the staging of NHL was IV A. The patient was treated, without surgical resection, only with imatinib 300 mg die obtaining after 4 month a good reduction of rectal mass and a subtotal remission of lymphadenopathy, confirmed by CT and PET. For the persistent reduction of platelets and white cells, caused by bone marrow infiltration , she started rituximab at dose 375 mg /m^2 weekly for four administrations and obtained a good response with improving of the peripheral blood cells. The revaluation after six month with bone marrow biopsy and PET showed a RC of both neoplasms. Actually the patient still follows therapy with imatinib 300 mg die and rituximab 375 mg/m^2 every three months. *Discussion.* The simultaneous NHL and GIST is very rare with an incidence of 3 per 10 billion people. GISTs are rare mesenchymal tumors that are mostly localized in the upper gastrointestinal tract and are frequently diagnosed in an advanced stage. Its localization at the rectum is rare. Conventional chemotherapy is ineffective. For resectable non-metastasized tumors surgical therapy is the treatment of choice. Imatinib is indicated in irresectable or metastasized GISTs. More than 80% of patients respond to imatinib therapy either with partial remission or stable disease. Imatinib is a "targeted" drug that inhibits the abnormal Bcr-Abl protein that causes chronic myelogenous leukemia; it is also approved for treatment of patients with . This drug might help to treat also T cell non-Hodgkin's lymphoma but the use in B NHL is very exceptional. *Conclusion.* This report is exceptional because there aren't in literature other similar cases described; the response of this NHL to imatinib is very interesting and could be a motivation for further studies in elderly patients.

PO-087

AN UNUSUAL CASE OF COMPOSITE LYMPHOMA: A SHORT CASE REPORT AND REVIEW OF THE LITERATURE

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Introduction. Composite lymphoma (CL) is a rare disease which is defined as the coexistence of two morphologically and phenotypically distinct types of lymphoid neoplasms occurring in a single anatomic organ or tissue with a low incidence, varying from 1% to 4.7%. The simultaneous occurrence of Hodgkin disease and non-Hodgkin lymphoma in a single lymph node is extremely rare. We present an unusual variant of composite lymphoma composed of nodular sclerosing Hodgkin lymphoma and diffuse large B-cell lymphoma in a single lymph node in a patient with a history of follicular small cleaved cell lymphoma occurs three years earlier. Etiology and pathogenesis are variable and complex. *Case report*. We report an unusual case of composite lymphoma arising in a 73-year-old woman with a history of follicular's lymphoma treated with 6 cycles of R-CHOP therapy 3 years before the current admission with RC and following follow-up negatives. The neoplasm was composed of LNH large cell lymphoma (CD79A+/BCL6-/CD10-/OCT2+/BOB1+/BOB1+/PU1+/KI67+) and nodular sclerosing Hodgkin disease (large vaguely nodular areas with characteristic lacunar-type Reed-Sternberg (RS) cells typical of nodular sclerosing HD were identified , CD30+, CD15+/-, CD79a-, PAX5+, CD3-, Ki67/MIB1 +++) within a single groin lymph node. Physical examination revealed a lymph nodes in more sites while a total computed tomographic scan was positive for lymph-adenopathy (III CS). The patient began treatment with PEGD protocol (Gemctitabine, cisplatin, etoposide and desametasone) and is in course the thirth cycle. Actually the patient is in clinical RC and she is going to revaluation with TC/PET total body. *Discussion*. CL is a separate types of non-Hodgkin's lymphoma or its rare association with Hodgkin's lymphoma within a single organ or tissue. The incidence of CL varied between 1-4.7%. The combination of 2 different types of

NHL composed of small cleaved cell lymphoma and diffuse large cell lymphoma is the most common variant, accounting for up to 58% of cases but the presence of composed of HD and one of the components of NHL in the same tissue, are very rare. According to our literature search, only six cases showing combination of classical Hodgkin lymphoma and DLBCL within the same site simultaneously were described. The clinical manifestation of CL is the same of ordinary lymphoma; the prognosis and the therapeutic decision depended by the unfavorable component of CL. *Conclusions.* CL with HD is rare and the pathogenesis is not clear. It must be carefully diagnosed, because the multiple disease entities may have entirely different natural histories, prognosis and treatment modalities. Also, careful study of such cases may clarify the possible pathogenic mechanisms of the interrelationship of clonal evolution in lymphoma.

PO-088

EARLY AND PERSISTENT TELOMERE SHORTENING IN LEUKOCYTES FROM PATIENTS TREATED WITH CONVENTIONAL CHEMOTHERAPY BUT NOT FROM THOSE EXPOSED TO RITUXIMAB ALONE

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Introduction. Patients with lymphoproliferative disease very often require treatments with chemotherapy. Recent reports suggest that the exposure of leukocytes to chemotherapeutic drugs may induce premature cell ageing. A good indicator of cell replication history is the length of telomeres. Indeed, telomere shortening has been documented in patients receiving chemotherapy. The loss of telomere sequences has been linked to the increased risk of developing secondary malignancy following chemotherapy. However, the cell type involved and the time course for the onset of chemotherapy-induced telomere shortening remain to be elucidated. In the present study changes in telomere length (TL) before and after cytotoxic drug exposure were evaluated. Main aims of the study were: i. to verify whether TL shortening is a phenomenon induced by extensive chemotherapy treatments or it may occur even after minimal drug exposures; ii. to define whether TL shortening following chemotherapy is reversible or permanent; iii. to investigate the different effect of chemoimmuno-therapy and immunotherapy alone.

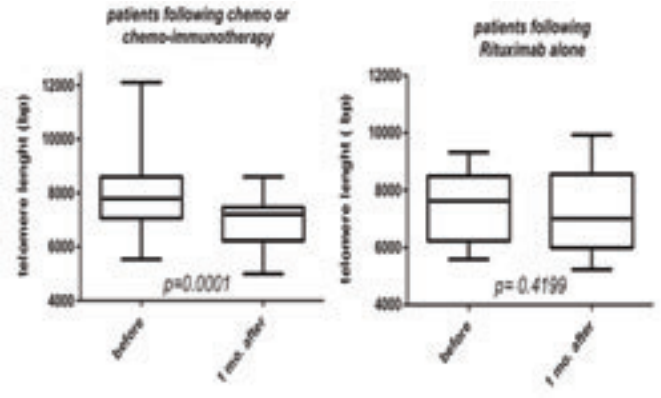

Figure 1. Changes of telomere in patients following chemo or chemo-immunotherapy and in patients treated with Rituximab alone.

Methods. Peripheral blood (PB) cells were obtained from 25 lymphoma patients undergoing chemotherapy-based treatments (16 R-CHOP, 3 ABVD, 1 BEACOPP, 3 R-Bendamustine, 1 R-MINE, 1 R-OXDHA) and 5 patients with primary immune thrombocytopenia (PTI) treated with the anti-CD20 Rituximab (R). Median age of patients was 55 years. All but three lymphoma patients were at their first treatment line. TL was assessed on granulocyte (GN), mononuclear cell (MNC) and on total leucocytes (total PB) before and after each chemotherapy course. In 10 lymphoma patients and in all PTI, TL was assessed also at long term since last therapy. TL was evaluated by southern-blot analysis. *Results.* A marked reduction in TL was detected in 22/25 (88%) patients undergoing conventional chemotherapy in all PB cells investigated. As shown in Figure 1, a marked TL loss following chemotherapy compared to pre-treatment values was observed in granulocyte (P=0.0001), although a TL reduction was detectable also in MNC (P=0.004) and total PB (P=0.001). In most patients TL shortening was detectable already after the first (21 pts) or the second (2 pts) chemotherapy course. In addition, TL shortening remained virtually unchanged up to 6 months since the last therapy in all 10 patients evaluated at long-term. No difference in TL was detected before and after drug exposure in the five patients receiving R monotherapy, even in patients followed up to 10 months since last R infusion (Figure 1). *Conclusions*. Results indicate that telomere shortening: i. can be detected in most patients as an early sign following chemotherapy; ii. is persistently detectable for several months since drug exposure; iii. can be most easily detectable in granulocytes; iv. is not detectable in a minority of patients receiving chemotherapy; v. is not observed in patients affected by PTI treated with Rituximab alone.

PO-089

NON-PEGYLATED LIPOSOMAL DOXORUBICIN OUTRUNS THE IMPACT OF THE DOSE-DENSE REGIMEN IN FRAIL ELDERLY PATIENTS WITH AGRESSIVE B-CELL NON HODGKIN LYMPHOMA. A MULTICENTRIC STUDY ON 129 PATIENTS

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The toxicity and efficacy of non-pegylated liposomal doxorubicin (NPLD) when substituted for conventional doxorubicin in the CHOP regimen were prospectively evaluated in 129 consecutive patients with newly diagnosed aggressive B-cell non-Hodgkin lymphoma (NHL). The median age of patients was 74 years (range: 65-89). At baseline 94/129 (73%) patients had stage III-IV disease, and 60/129 (46%) patients had an intermediate or high risk International Prognostic Index. Median performance status was WHO 1 (range 0-3); median number of comorbities was 2 (range 1-6). The median left ventricular ejection fraction (LVEF) before chemotherapy was 59% (range 40-73). Patients were split in 2 groups according to a functional assessment of frailty, Activities of Daily Living (ADL). Thirty-nine patients with an ADL=6 were addressed to receive dose-dense R-COMP every 2 weeks (14), whereas 90 patients with an ADL<6 were addressed to receive R-COMP every 3 weeks (21). All the variables were comparable between the 2 groups (14 vs 21). A total of 715 cycles of chemotherapy were administered (234 R-COMP 14 and 481 R-COMP 21). All patients were evaluable for response. The overall response rate was 89% (complete response 73%, partial response 16%). With a median follow-up of 24 months (range 2-30) as of January 2012, 87/129 patients (67%) are alive and disease free, whereas 32/129 (25%) are dead and 8/129 (6%) are alive with active disease. Log rank analysis showed high risk IPI (4/5; P=0.02) to be a variable predictive for shorter event-free survival (EFS), whereas again high risk IPI (4/5; P=0.04), age >70 years (P=0.009), advanced-stage disease (stage III-IV; P=0.01) and performance status 2-3 (P=0.003) were all predictive of shorter overall survival (OS). The Cox proportional hazards regression model identified age >70 years (P=0.03) and perfomance status 2-3 (P=0.02) as the variables with a negative impact on OS. Finally, no statistically significant difference in terms of response was observed within the two groups (14 vs 21). Toxicity was mainly hematological in both groups. Grade 3/4 neutropenia occurred in 11% and 22% of cycles in the R-COMP 14 and 21 groups respectively, with an incidence of febrile neutropenia of 3% and 8% respectively. The relative dose intensity for the regimens was 93% for the R-COMP 14 group and 90% for the R-COMP 21 group, respectively. Regarding cardiotoxicity, only 8/129 patients presented a grade II-IV WHO toxicity. Up to now, this is the largest series of frail elderly patients with aggressive NHL treated with NPLD instead of conventional doxorubicin reported. Our data strongly suggest that NPLD induces a high rate of long-lasting complete response (CR) in a population of frail elderly patients not suitable for a treatment with conventional anthracyclines. In conclusion, the overlapping rate of CR between the dose-dense R-COMP 14 group and the standard R-

COMP 21 group suggest that the use of NPLD outruns the impact of a dose-dense regimen in a substantial proportion of frail elderly patients, representing a therapeutic opportunity for a category of patients not suitable for a dose-dense treatment. *Acknowledgments*. Supported in part by AIL Pesaro Onlus.

PO-090

THE PI3K/ERK DUAL INHIBITOR AEZS-136 TRIGGERS A POTENT ANTITUMOR ACTIVITY AGAINST HODGKIN LYMPHOMA CELL LINES THROUGH A ROS-DEPENDENT MECHANISM

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Introduction. A significant proportion of Hodgkin lymphoma (HL) patients refractory to first-line chemotherapy or relapsing after autologous transplantation are not cured with currently available treatments and require new treatments. The RAS/RAF/MEK/ERK (MAPK) and the PI3K/Akt signaling pathways promote cellular proliferation and survival. In many tumors, including HL, molecular events induce constitutive activation of both pathways strongly suggesting the therapeutic relevance of targeting dysregulated pathways. Indeed, feedback loops and cross-talk between pathways limit the clinical effectiveness of selectively targeting individual pathways. In contrast, dual or multiple-targeted approaches might have a relevant clinical impact in anticancer therapy. In the present study, we used the dual PI3K/Erk inhibitor AEZS-136 (Æterna Zentaris GmbH, Germany, EU) to investigate the *in vitro* cytotoxic activity and mechanism(s) of action of this drug in HL cell lines. *Methods.* Four HL cell lines (L-540, SUP-HD1, KM-H2 and L-428) were used to investigate the effects of AEZS-136 by means of *in vitro* assays analyzing cell growth and cell death. Additionally, live cell imaging was used to asses the production of reactive oxygen species (ROS), and Western blotting (WB) to assess modulating effects of AEZS-136 on MAPK, PI3K/AKT as well as apoptosis pathways. *Results.* Exposure of all HL cell lines to increasing concentrations of AEZS-136 (2.5 - 15 μM) resulted in a time and dose-dependent cell growth inhibition (range, 30% to 80%). Cell cycle analysis indicated that treatment with AEZS-136 (10 μM) significantly reduced the percentage of cells in S-phase by 33 to 13% on average and increased the percentage of G0/G1-phase by 47 to 68% on average. Significant levels of apoptosis were only observed for L-540 (14±3% vs 62±9%, P≤.0001) and SUP-HD1 (15±2% vs 46±2%, P≤.0001) cell lines associated with severe mitochondrial dysfunction (up to 40%, P≤.001). Apoptosis was caspase-independent by WB analysis and blocking experiments using the pan-caspase inhibitor Z-VADfmk. WB analysis showed that the cytotoxic activity of AEZS-136 was associated with dephosphorylation of MAPK and PI3K/Akt pathways. Interestingly, upon AEZS-136 exposure, three out of four cell lines (L-540, SUP-HD1 and KM-H2) showed a significant increase in JNK phosphorylation associated with a pronounced time-dependent generation of ROS. *Conclusions.* Dual targeting of MAPK and PI3K/Akt pathways using AEZS-136 inhibitor is associated with a potent anti-lymphoma activity. These findings deserve further investigation *in vivo* in xenograft models of HL.

PO-091

RITUXIMAB AND BENDAMUSTINE THERAPY IN PATIENTS WITH RELAPSED OR REFRACTORY WALDENSTRÖM'S MACROGLOBULINEMIA (WM)

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Background. WM is an incurable disease, with an overall medial survival of only 5-6 years. First-line therapy of WM has been based on single-agent or combination therapy with alkylator agents (e.g. chlorambucil or cyclophasphamide), nucleoside analogues (cladribine or fludarabine), and the monoclonal antibody rituximab. Novel therapeutic agents that have demonstrated efficacy in WM include thalidomide, lenalidomide, bortezomib, everolimus and bendamustine. *Methods.* We report the treatment outcome for 6 relapsed/refractory Waldenström's macroglobulinemia (WM) patients. Treatment consisted of bendamustine (90 mg/m^2 I.V. on days 2, 3) and rituximab (375 mg/m^2 I.V. on day 1) for all patients. One rituximab-intolerant patient received bendamustine alone. Each cycle was 4 weeks, and median number of treatment cycles was 4. *Results.* The clinical stage (remission, progression or stable disease) was defined with clinical re-evaluation after chemotherapy and/or re-staging 6 months after end of therapy. At best response, median serum IgM declined from 3500 to 500 mg/dL, and hematocrit rose from 29.9% to 37.8%. Overall response rate was 83.3%, with 4 VGPR and 1 PR. Overall therapy was well tolerated. Prolonged myelosuppression was more common in patients who received prior nucleoside analogues. *Conclusions.* Bendamustine in combination with Rituximab demonstrates an excellent effectiveness in previously treated WM patients, with an acceptable toxicity profile. These agents, when compared to traditional chemotherapeutic agents, may lead in the future to higher responses, longer remissions and better quality of life for patients with WM.

PO-092

CD200 ANTIGEN IN B-CELL DERIVED NEOPLASMS: EXPRESSION AND CLINICAL IMPACT IN 77 CONSECUTIVE PATIENTS OF A SINGLE ISTITUTION

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Introduction. CD200 is a cell surface molecule with immune-regulatory function consistently expressed on lymphoid neoplastic cells. CD200antigen has been found in case studies with a frequency that ranges from 20 % in follicular lymphoma to almost100 % of B-CLL. The interest of CD200 protein from a practical point of view is for its diagnostic and potentially prognostic applications. As diagnostic marker it is useful for the distinction between MCL and B-CLL . In fact, in mantle cell lymphoma, CD200 is either generally absent or expressed at a low intensity by a subset of pathological cells; on the contrary, it is always present in B-CLL cells. Furthermore, there are some reports about very frequent CD200 expression in HCL. Prognostic significance has been described in plasmacells neoplasms. Another important aspect of CD200 study, is represented by potentially therapeutic impact such as targeted-therapy. *Methods.* We carried out a study on 77 consecutive patients with B-lymphoproliferative disorders diagnosed over a period of 2 years (2010 to 2012). We analyzed immunophenotype of B-cells of lymphoproliferative disorders by multiparameter flow cytometry. The following monoclonal antibody panel was used: CD19, CD5, CD23, CD20, CD38, CD10, FMC7, CD22, CD103, CD25, CD11c, CD200, CD79b, CD49d, CD3, CD4, CD8. We checked the expression of specific surface marker CD200. According to mean fluorescence intensity, we identified 3 distinguishable CD200 subsets: CD200 neg, CD200dim, and CD200bright. CD200 was considered negative if the population's peak fluorescence was localized under 101, CD200 was considered "positive bright" if the population's peak fluorescence was localized upper 102 and CD200 was considered "positive dim" if it was localized between 101 and 102. *Results.* The distinct lymphoproliferative diseases were as follow: 41 B-LLC, 8 SMZL, 4 MCL, 4 HCL, 3 FL, 8 DLBCL, 2 LLP, 6 non otherwise specified low-grade B-lymphoma. The pattern of expression of CD200 antigen was: positive in all case of B-CLL (8 CD200dim, 33 CD200bright); positive with "bright" expression in all case of HCL and in all case of FL; 4/5 negative in MCL; 7/8 negative in SMZL; "dim" positive in all case of LLP; 4/8 "bright" positive, 2/8 "dim" positive and 2/8 negative in DLBCL; 2/6 "bright" positive and 4/6 "dim" positive in the low-grade lymphoma group. A trend toward a "dim" expression of CD200 was observed in subjects with B-CCL with low burden of disease. The "CD200 positive" case of MCL was associated with a primary splenic form, with a more favorable prognosis. *Conclusions.* In the immunophenotypic characterization of B-lymphoproliferative diseases, CD200 can be included in the routine antigens panel especially for the identification of different CD19/CD5 disorders. Otherwise it could represented a parameter that correlate with "indolent" or "aggressive" clinical course of B-CLL and MCL.

PO-093

LYMPH NODE STROMAL CELLS FROM NON HODGKIN LYMPHOMAS REGULATE GAMMADELTA T CELL FUNCTIONS: RESCUE BY AMINOBIPHOSPHONATES

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Introduction. Gammadelta (gd) T lymphocytes are involved in stress response to infected or transformed tissues through the recognition of: i) unprocessed non-peptide molecules, namely phosphoantigens (PAg) derived via the mevalonate or the 1-deoxy-D-xylolose-5-phosphate pathway in mammalian or bacterial cells; ii) stress-inducible MHC-class I related MIC-A/MIC-B molecules, and UL16-binding proteins (ULBPs) induced at the cell surface by viral infections or tumor transformation; iii) antibody-opsonized cells or microrganisms through the binding of IgG Fc by the Fcgamma receptor IIIA (CD16), which mediates the antibody-dependent cell cytotoxicity (ADCC). gdT lymphocytes are involved in the surveillance against various hematological malignancies including chronic lymphocytic leukemias, Hodgkin (HL) and non-Hodgkin lymphomas (NHL), by the mean of one or another of the above mentioned mechanisms. *Methods.* 48 patients with NHL (30 follicular and 18 diffuse large B cell lymphomas) were analyzed. Lymph node (LN) biopsies were obtained under diagnostic procedures, provided informed consent and approval by the institutional ethical commettee and used for phenotypic, functional and molecular studies, compared to 15 healthy LN, taken as sentinel LN and resulted free of neoplastic disease. Lymph node mesenchymal stromal cells (LNMSC) were obtained by culturing LN cell suspensions; gdT lymphocytes were obtained from peripheral blood and from LN. The following experimental procedures have been performed: i) co-culture of gdT cells with LNMSC; ii) cytotoxicity against lymphoma cells and ADCC with anti-CD20 antibody Rituximab; iii) gdT cell phenotype, proliferation, cytokine production (TGFbeta, TNFalfa, IFNgamma, IL10) and release (by cytofluorimetry and ELISA); iv) expression of different cytokines in situ by Q-RT-PCR. *Results.* we show that: i) LNMSC can inhibit NKG2D-mediated lymphoma cell killing but not Rituximab-mediated ADCC; ii) pre-treatment of LNMSC with the aminobiphosphonates pamidronate or zolendronate can rescue the ability of gdT cells to recognize lymphoma cells via NKG2D iii) the rescue is due to inhibition of TGFbeta (that downregulates NKG2D expression) and increase in IL15 (that upregulates NKG2D) production by LNMSC; iv) zolendronate-treated LNMSC drive gdT lymphocyte differentiation into effector memory (EM) T cells, producing Th1-type cytokines, rather than IL10 that might contribute, together with TGFb, to the expansion of regulatory T cells (Treg). *Conclusions.* in NHL the LN microenvironment, in particular through the contribution of LNMSC, leads gdT lymphocytes to differentiate into either Th1 or Treg, depending on the prominent cytokine milieu produced by LNMSC. Drugs able to trigger gdT cell recognition of PAg, can push the balance towards the first type of differentiation, rescuing the recognition and killing of lymphoma cells through NKG2D. This may contribute with Rituximab-mediated ADCC to elimination of lymphoma cells.

PO-094

IN VITRO AND *IN VIVO* MODEL OF EBV-POSITIVE ACTIVATED DIFFUSE LARGE B- CELL LYMPHOMA WITH PLASMACYTIC DIFFERENTIATION

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Introduction. Small B-cell neoplasms can show plasmacytic differentiation and may potentially progress to aggressive lymphoma. Diffuse Large B cell Lymphoma (DLBCL) represents a very heterogeneous group of aggressive lymphomas. Some DLBCLs show the phenotype of terminal B-cell differentiation, representing a continuum spectrum of lesions that ranges from conventional activated DLBCL to plasma cell disorders. The lack of representative cell lines and animal models is a major impairment in understanding the biology of DLBCL's subtypes. Immortalization of malignant cells is often due to EBV infection. We established VR09 cell line, an EBV-positive DLBCL cell line with plasmacytic differentiation, obtained from a case of atypical B-cell chronic lymphoproliferative disease with plasmacytic features, having a tumorigenic potential *in vivo* into Rag2-/- -chain-/- mice. *Methods.* Mononuclear cells from human DLBCL bone marrow, were seeded in RPMI 10% FBS medium. Cells showed spontaneous proliferative capacity and a cell line was established after months of continuous culture. Cells were evaluated by flow cytometry, immunohistochemistry, molecular biology and fluorescence in situ hybridization (FISH). Cells were inoculated subcutaneously into 6 immunodeficient Rag2-/- -chain-/- mice. Subcutaneously-growing tumors were evaluated by immunohistochemistry and FISH. Disaggregated cells from masses were cultured again to confirm their biological features and proliferative capacity. *Results.* cells in suspension formed large clumps with round shape and they revealed a plasmacytic/plasmablastic appearance. When subcutaneously injected in mice, they grew as spherical tumors and maintained their proliferative capacity once disaggregated from mass and cultured. Evaluated by flow cytometry and immunohistochemistry, VR09 cell line and tumors displayed the phenotype of activated stage of B cell maturation, with secretory differentiation (CD19+ CD20+ CD79a+ CD79b+/- CD138+ cyclin D1- Ki67 80% IgM+ IgD+ MUM1+ MNDA+ CD10- CD22+ CD23+ CD43+ K+, - Bcl2+ Bcl6-) and they presented episomal EBV genome, chromosome 12 trisomy, absence of c-MYC rearrangement, presence of somatic hypermutation in the VH region, variants of Card 11 and CD79B genes and wild-type p53. *Conclusions.* This model could be useful to further characterize activated DLBCL with plasmacytic features and for further studies about the development of high-grade non Hodgkin Lymphoma in patients with low-grade B-cell lymphoproliferative disorders with plasmacytic differentiation, that is a rare but possible event in clinical practice.

PO-095

MYELOID IMPAIRMENT HAS COMPARABLE PREDICTIVE VALUE OF INTERIM-PET IN HODGKIN'S LYMPHOMA

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Introduction. Combination chemotherapy with ABVD (doxorubicin, bleomycin, vinblastine, and dacarbazine is considered the gold standard for Hodgkin`s lymphoma (HL), but a small fraction of patients fails to achieve long term disease control for either resistant or relapsing disease. The most promising intensive regimen BEACOPP (bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, and prednisone) has been proposed for a better disease control, despite immediate and long term side effects. Thus, new biomarkers to identify risk-adapted strategies are an emerging need. Among these, interim-PET after two courses of chemotherapy (PET-2)and ratio between absolute lymphocyte and monocytes count ALC/AMC in peripheral blood at diagnosis have been suggested. We previously described that a circulating sub-population of immunosuppressive myeloid cells (MDSC) have the ability to suppress T-cell immune response, and have a prognostic value in predicting interim-PET positivity or early relapse. In this retrospective study we evaluated sensibility and specificity of interim-PET, ALC/AMC and MDSC-count in peripheral blood at diagnosis to predict progression free survival (PFS). *Methods.* From September 2008 to October 2011, 52 consecutive patients affected by classical HL were evaluated, treated with standard ABVD therapy. In peripheral blood, we evaluated ALC/AMC ratio and circulating levels of immature im-MDSC by flow cytometry defined as CD45+, CD34+, CD11b+, CD13+, CD14- and arginase1 expression in neutrophils, lymphocytes and monocytes at diagnosis, after interim PET and at the end of treatment. *Results.* After a median follow-up of 19.2 months (range, 3.5-36.4 months), 44 patients (84.6%) were in continued complete remission (cCR), 8 failed treatment (15.4%) after a median of 11.2 months. PET-2 was available for all patients. 6/52 patients (11.5%) were PET-2 positive and all of them shifted to BEACOPP scheme. Despite this risk-adapted strategy, four patients showed treatment failure (progression/relapse). PET-2 had

sensitivity for predicting 2-year PFS of 50% (95% CI%, 15.7 to 84.3%) and a specificity of 95.5% (95% CI, 84.5% to 99.4%).48/52 patients had high ALC/AMC-DX ratio>1.1, defined in accord to [1], with sensitivity for predicting 2-year PFS of 93.8% (95% CI, 69.8 to 99.8), but a low specificity of 9.1% (95% CI, 4.2% to 16.5%). HL patients at diagnosis showed higher levels of im-MDSC when compared to matched for sex and age healthy controls (3.02±0.25 vs 1.62±0.16, P<0.0001), with return to normal values within the first 2 cycles of chemotherapy (1.81±0.31, P=0.013). im-MDSC count had sensitivity for predicting 2-year PFS of 75% (95% CI%, 34.9 to 96.8%) and a specificity of 86.4% (95% CI, 72.7% to 94.8%).All HD patients exhibited an increased amount (100-500 folds) of Arginase1 mRNA in both neutrophils and monocytes (P<0.001), with a significant decrease during the treatment (20-50 folds, P<0.001). *Conclusion.* A low ALC/AMC-DX ratio correlates with a good prognosis, but in our series we were not able to confirm the high specificity of this marker and its predicting value was certainly inferior to PET-2. im-MDSC count had high sensitivity and specificity for predicting 2-year PFS.

PO-096

HETEROGENEITY OF THE VESSEL WALL IN PRIMARY HUMAN CENTRAL NERVOUS SYSTEM LYMPHOMAS

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Background. No literature data are available concerning the phenotypic characteristics of the vessel wall in primary central nervous system lymphomas. Aims and Methods. We have investigated the phenotypic features of the vessel wall in 24 human diffuse large B-cell primary central nervous system lymphomas by means of immunocytochemistry and confocal laser microscopy. *Results*. i) A high aquaporin-4 expression correlated with a high Ki-67 index and aquaporin-4 marked tumor and endothelial cells in cytoplasm and plasma membranes, while aquaporin-4 expression was low in tumor areas with a low Ki-67 index where few tumor cells were positive to aquaporin-4, and endothelial cells showed aquaporin-4 expression on their abluminal side. ii) Different type of cells participated to vessels formation: CD20+ tumor cells and factor VIII+ endothelial cells; aquaporin-4+ tumor cells and CD31+ endothelial cells; CD20+ and aquaporin-4+ tumor cells; glial fibrillary acidid protein+ endothelial cells surrounded by glial fibrillary acidic protein+ tumor cells; endothelial precursor cells CD133+/CD31+ and Nestin+/CD31+. *Conclusions.* Overall, these data documented that tumor microvasculature in lymphomas is extremely heterogeneous, and confirm the importance of neo-angiogenesis in their pathogenesis.

PO-097

ROLE OF WHOLE BODY MAGNETIC RESONANCE INCLUDING DIFFUSION WEIGHTED IMAGING IN THE INITIAL STAGING OF LYMPHOMA

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Introduction. The diagnostic workup usually employed to stage patients with Hodgkin (HL) and non-Hodgkin lymphoma (NHL) at diagnosis is based on Multi Detector Computed Tomography (MDCT) and, in most instances, 18F-Fluorodeoxyglucose Positron Emission Tomography co-registered with low dose Computed Tomography (18F-FDG PET-CT). Both these imaging techniques require the delivery of considerable dose of ionizing radiation. This is of particular concern in young patients, in fertile women and, in general, in patients requiring repeated diagnostic procedures during treatment and follow up. Indeed, extensive use of radiation-based diagnostic procedures may concur to the increased risk of secondary malignancies observed in lymphoma patients following treatment. Whole body Magnetic Resonance Imaging (Wb-MRI) is an emerging technique that allow to avoid ionizing radiation, while providing accurate anatomical information. Moreover, the development of the "diffusion weighted" (DWI) technique has made it possible detailed morpho-functional evaluation. Aim of this study was to evaluate feasibility and diagnostic accuracy of MDCT and Wb-MRI with and without DWI in the initial staging of PET+ve lymphoma. *Methods.* We retrospectively evaluated 15 patients with newly diagnosed and histological proven lymphoma (7 HL and 8 NHL, all with documented FDG avid disease). All patients underwent the usual staging procedures, including bone marrow biopsy, 18F-FDG PET-CT, MDCT, and Wb-MRI-DWI as well. All procedures were performed soon before starting treatment. According to the Ann Arbor staging classification, stage I was diagnosed in 7% of patients, stage II in 13%, stage III in 40% and stage IV in 40%. Both MDCT and Wb-MRI, with and without DWI, were independently evaluated by two expert radiologists, without knowing the results of the other diagnostic procedures. *Results.* MDCT findings were accordant with 18F-FDG PET-CT results in 80% of cases. The agreement of WB-MRI with 18F-FDG PET-CT was as high as 87%. When the DWI analysis was added, the rate of agreement raised to 93%. Based on MDCT results, three patients were incorrectly staged, i.e. 2 patients "under-staged" and 1 with over-staging; on the other hand, Wb-MRI-DWI incorrectly understaged 1 patient only. In 4/15 cases (27%) MDCT was unable to identify bone lymphomatous localizations, with subsequently under-staging in 2 of them. Wb-MRI-DWI detected bone lesions in 100% of cases. *Conclusions.* Wb-MRI including DWI is a non invasive and non-radiation based technique that provides useful anatomic and functional information. The results of this analysis demonstrate the possible use of Wb-MRI as part of the initial diagnostic workup in malignant lymphoma. In addition, the study gives further support to the value Wb-MRI as an alternative procedure to MDCT. Lastly, due to its high detection capacity, Wb-MRI-DWI should be considered as an effective tool for the accurate diagnostic evaluation in non-FDG avid lymphoma.

PO-098

ANEMIA IN DIFFUSE LARGE B CELL NON-HODGKIN LYMPHOMA: THE ROLE OF IL-6, HEPCIDIN AND ERYTHROPOIETIN

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Introduction. Anemia is a frequent sign in patients with DLBCL at diagnosis, and it is associated with unfavourable patient characteristics. Multiple factors may contribute to anemia in DLBCL. We studied the contribution of some principal players for erythropoiesis to the development of anemia in DLBCL: the iron-store regulator hepcidin, the hormone erythropoietin, and the inflammatory cytokine IL-6. *Methods.* We studied 53 patients with DLBCL (median age 61 years, range 16-78 years; 28 females and 25 males). A group of 24 healthy individuals (median age 41 years, range 18-63 years; 13 female, 11 males) was used as control. Plasma samples were analyzed for hepcidin levels using a combination of weak cation exchange chromatography and time-of-flight mass spectrometry (TOF MS); IL-6 and erythropoietin levels were determined using ELISAs. Associations with patient characteristics were analyzed using standard statistics (STATA 10). *Results.* At diagnosis, 34 of 53 (64%) DLBCL patients were anemic defined by Hb levels <13 g/dL (males) or <12 g/dL (females) with most patients having mild anemia. As expected, haemoglobin concentration was lower in patients with B-symptoms, and in patients with age-adjusted IPI score >1. There was no significant difference in haemoglobin levels between patients with or without bone marrow infiltration. Hepcidin plasma levels were significantly higher in patients compared to controls (P=0.006), and in patients with more aggressive disease as elevated LDH levels (P=0.0004), presence of B-symptoms (P=0.07), and age-adjusted IPI score > 1 (P=0.01), independent of the presence of anemia. Hepcidin strongly correlated to ferritin (r=0.77, P<0.0001) and to IL-6 (r=0.30, P=0.03) concentration and inversely correlated to iron-binding capacity (r=-0.36, P=0.04), but not to haemoglobin and erythropoietin values. The majority of patients showed defective endogenous erythropoietin production, as indicated by an observed/predicted (O/P) ratio less than 0.8, particularly when anemia was present (P=0.01). IL-6 inversely correlated to haemoglobin values both in univariate (r=-0.35, P=0.009) and was the only param-

eter in multivariate analysis, including also hepcidin and erythropoietin that correlated to haemoglobin levels (P=0.04). *Conclusion.* Our findings suggest that in DLBCL elevated hepcidin levels resulting into iron-restriction, and inadequate erythropoietin response are frequent, but that elevated IL-6 levels may play the major role for the development of anemia.

PO-099

BRENTUXIMAB SALVAGE THERAPY IN HODGKIN LYMPHOMA PATIENTS RELAPSED AFTER ALLOGENEIC STEM CELL TRANSPLANTATION: A SINGLE CENTRE EXPERIENCE

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Introduction. Hodgkin's Lymphoma (HL) has a poor prognosis especially if relapse occurs after allogeneic stem cell transplantation (allo-SCT). HL is one of the most common tumors expressing CD30 and CD30-directed therapy can be an effective choice to enhance the anti-tumor activity in refractory disease. *Methods.* Three patients with CD30-postive HL were submitted to allo-SCT: they were in complete response according to International Workshop Criteria (IWC) with a negative positron emission tomography (PET) (n=1) and partial response according to IWC with a positive PET at the site of previously involved nodal masses (n=2). Patients were conditioned with reduced intensity conditioning. The main characteristics about conditioning regimen and stem cell source are reported in Table 1. At the 3th month after allo-SCT 2 out of 3 patients were PET negative. During post allo-SCT follow up we observed two histologically confirmed relapse respectively on +3 and +7 month and one progressive disease on +8 month. As salvage therapy before anti CD30 specific therapy(Brentuximab), two patients were treated with systemic chemotherapy to reduce the disease burden: only one of these patients showed a partial response as the other showed a progressive disease. Brentuximab at a dose of 1,8 mg/Kg was started in all three patients at +30, 11 and 5 months from allo-SCT (Table 1). *Results.* One patient with partial response, after conventional chemotherapy, received five cycles and three donor lymphocyte infusion (DLI) with achievement of complete response. The patient developed a Guillain Barrè syndrome after administration of three Brentuximab and two DLI and was treated with intravenous immunoglobulin. Another patient with progressive disease, after conventional chemotherapy, received nine cycles and a partial response was documented after the fourth. The third patient with relapse disease after allo-SCT received only two Brentuximab cycles until now and response is under evaluation. No hematologic toxicity was documented. In conclusion we observed tumor regression in two patients with mild-moderate toxic effects in only one patient. *Conclusions.* Our preliminary data suggest that Brentuximab is a valid therapy in patients with CD30-positive HL that are refractory or relapsed after allo-SCT. A synergism between Brentuximab mechanism of action and immunological graft versus lymphoma may induce durable objective response with a possible crucial role in disease eradication. The possible interaction with the development of autoimmune disorder such as Guillain Barrè syndrome require further studies.

Table 1. Patients disease status and therapy.

Acute Leukemias 2

PO-100

CLOFARABINE FOLLOWED BY CYCLOPHOSPHAMIDE FOR TREATMENT OF RELAPSED/REFRACTORY ACUTE LYMPHOBLASTIC LEUKEMIA IN ADULT PATIENTS

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Introduction. Relapsed or refractory adult acute lymphoblastic leukemias (ALL) have poor prognosis. The strategy for treating these patients is through reinduction chemotherapy followed by allogeneic stem cell transplantation, provided that the toxicity of the salvage regimen is acceptable. Clofarabine, a next-generation deoxyadenosine analog, has demonstrated significant activity in children and adults with refractory lymphoid and myeloid leukemia in early clinical trials and was granted approval for use in children with acute lymphoblastic leukemia in second or higher relapse. Promising activity of clofarabine in combination with cyclophosphamide, with DNA damage and apoptosis in both AML and ALL blasts, has been reported (Karp JE et al, Blood 2007). *Aim.* We present a series of ten cases in which clofarabine was combined with cyclophosphamide in adult patients with relapsed or refractory acute lymphoblastic leukemia. *Methods.* Patients aged 23-59 years with refractory/relapsed ALL were treated at the dose of clofarabine 10 mg/m^2 + cyclophosphamide 400g/m^2 on days 1-3 and 8-10. We evaluated the overall remission rate (ORR), duration of remission (DOR) and overall survival (OS). Minimal residual disease (MRD) by molecular targeting was considered in all patients. *Results.* Nine patients received clofarabine 10 mg/m^2 + cyclophosphamide 400 mg/m^2, both on Days 1–5 and 8-10;one patient received only one cycle. All patients had relapsed/refractory lymphoblastic leukemia and had received multiple priors tharapies. Eight had pre-B cell ALL, 2 pts had T cell ALL; two pts had received a prior hematopoietic stem cell transplant (HSCT). Four patients achieved a morphologic complete remission (CR); two patients went on to receive allogeneic transplants after clofarabine/cyclophosphamide salvage. The median of Overal survival (OS) for all the patients was 103 days, the media was 172,70days. The overall remission rate (ORR) was 44,4%, and we estimated a duration of remission (DOR) as 223,25 days in media (we calculated from the first day of remission). Treatment was complicated by neutropenic fever (n=4), grade III-IV mucositis (n=3), prolonged aplasia >30 days (n=3). One patient died of sepsis before completing the regimen. *Conclusion.* Combination treatment with clofarabine and cyclophosphamide in adults pts with refractory or relapsed ALL resulted in an ORR of 44%, two pts proceeded to HSCT. The safety profile is acceptable in this relapsed/refractory population. The response rates and durability of remission observed with this regimen were encouraging given that these patients were highly refractory to prior therapies.More studies with this combination in adults are warranted.

Table 1. Characteristics of the patients.

N	Age	Diagnosis	Previous regimen (n.)	Response after Clofara-Cy	Toxicities	HSCT
1	35	B-ALL	3	Refractory	Neutropenic fever	n
2	57	B-ALL	4	Refractory	Neutropenic fever	n
3	52	B-ALL common	4+HSCT	Refractory	Prolonged marrow aplasia	n
4	23	pre T-ALL	4	Refractory	mucositis	n
5	27	B-ALL	4	Complete remission	mucositis	y
6	59	B-ALL common	3	Complete remission	Prolonged marrow aplasia	y
7	40	B-ALL	2+HSCT	Complete remission	Neutropenic fever	n
8	57	T-ALL	2	Refractory	mucositis	n
9	57	B-ALL common	1	Complete remission	Neutropenic fever	n
10		B-ALL	3	Refractory	Prolonged marrow aplasia	n

PO-101

CLOFARABINE IN COMBINATION WITH CYTARABINE (ARA-C) FOR TREATMENT OF RELAPSED/REFRACTORY ACUTE MYELOID LEUKEMIA IN ADULT PATIENTS

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Introduction. Relapsed/refractory AML patients have a poor prognosis, with CR rates of 1%-30%, unless allogeneic hematopoietic stem cell transplantation (HSCT) is an available option. Although retrospective modeling studies have demonstrated the prognostic value of selected parameters, responses with salvage therapies remain still poor. It was previously established the activity of clofarabine plus cytarabine in AML relapse (clofarabine dosed once daily for 5 days with 40 mg/m^2 followed 4 hours later by ara-C at 1 g/m^2 per day). However, modifications of this combination in AML therapy of relapsed/refractory patients warrant further evaluation. *Aim.* To determine the efficacy and safety of clofarabine and cytarabine (Ara-C) in adult patients with relapsed or refractory acute myeloid leukemia (AML). *Methods.* Patients aged 35-66 years with refractory/relapsed AML were treated at the dose of clofarabine 30 mg/mq on days 1-5 + cytarabine 1000 mg/mq gg on days 1-5. We evaluated the complete remission rate (CRR), duration of remission (DOR) and overall survival (OS). Minimal residual disease (MRD) by molecular targeting was considered in all patients.

Table 1. Characteristics of the patients.

N	Age	Refractory/ relapsed	Karyotype and molecular genetyc	Previous regimen (n.)	Response after Clofarabine/ ARA-C	Toxicities	HSCT
1	55	refractory	47 XY,+8;	1	Refractory	Severe febrile neutropenia	n
2	41	refractory	48 XY, -21,+ 3 der(21)	1	Complete remission	Severe febrile neutropenia	n
3	46	relapsed	46,XX/FLT3 ITD mutated	2	Refractory	Severe febrile neutropenia	n
4	55	relapsed	46, XY	2	Complete remission	Severe febrile neutropenia	y
5	62	refractory	Complex karyotype, monosomy 7	1	Refractory	skin rash	n
6	66	refractory	47,XX,+8	1	Refractory	Severe febrile neutropenia	n
7	49	refractory	46 XX	2	Complete remission	Severe febrile neutropenia	y
8	53	relapsed	46,XY	1	Died in induction	Skin rash	n
9	62	relapsed	46, XY	3	Complete remission	Nausea, vomiting	n
10	48	relapsed	46,XY+8	3+HSCT	Complete remission	Nausea, vomiting	n
11	40	refractory	48,XY,+8	2	Died in induction	Nausea, vomiting	n
12	50	relapsed	46, XY	2	Refractory	Mucositis	n
13	42	refractory	46, XX/FLT3/ ITD mutated	1	Complete remission	Mucositis	n
14	61	relapsed	46, XY	1	Refractory	Severe febrile neutropenia	n
15	35	relapsed	46, XX	1	Complete remission	Severe febrile neutropenia	y
16	40	relapsed	46, XY	1	Complete remission	Severe febrile neutropenia	y
17	54	relapsed	46, XX/FLT3/ ITD mutated	2+HSCT	Complete remission	Hepatic transaminase elevations	n
18	62	refractory	46, XY	1	Complete remission	Severe febrile neutropenia	n

Results. Eighteen patients received clofarabine 30 mg/mq on days 1-5 + cytarabine 1000 mg/mq gg on days 1-5 (their characteristics are summarized in Table 1), followed by gentuzumab therapy in only three patients. All patients had relapsed/refractory myeloid leukemia and had received multiple priors tharapies. Two pts had received a prior hematopoietic stem cell transplant (HSCT). Nine patients achieved a morphologic complete remission (CR); four patients went on to receive allogeneic transplants after clofarabine/ARA-C salvage. The complete remission rate (CRR) was 55,56%.The Median of Overall survival for all patients was 53 days (range 23-769), while the media of Overall survival (OS) was 152.22 days, and we estimated a duration of remission (DOR) as 75.00 days in median (range 3-785), and 232,67 days in media (we calculated from the first day of remission). Treatment was complicated by neutropenic fever (n=10), grade III-IV mucositis (n=2) , skin rush (n=2) grade II- III, hepatic transaminase elevations (n=1). Two patient died of sepsis during the induction. *Conclusion.* Combination treatment with clofarabine 30 mg/mq and ARA-C 1000 mg/mq in adults pts with refractory or relapsed AML resulted in an CRR of 55,56 % and of the 10 patients who achieved a CR, four (40%) proceeded to HSCT (two are still alive and in complete remission). The safety profile is acceptable in this relapsed/refractory population, and our results are very similar to previous regimes using higher clofarabine dosages. More studies with this combination in adults are warranted.

PO-102

IMMUNOPHENOTYPIC ANALYSIS OF THE STEM CELL COMPARTMENT IN PATIENTS WITH ACUTE MYELOID LEUKEMIA (AML)

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Introduction. Acute leukemias are the result of neoplastic transformation of hematopoietic stem cells (HSCs). Recent studies showed that immature cells with the biological features of stem cells can be identified in leukemia and are responsible for tumor development, maintenance and relapse, as they have the ability to self-renew and intrinsically resist to drugs, similarly to stem cells. The presence of leukemia stem cells (LSCs) has been shown mainly in the bone marrow (BM) CD34+/CD38- cell compartment of patients with AML, in this same cell fraction where normally HSCs can be detected in healthy subjects. Aim of this study was to identify specific phenotypic abnormalities of LSCs that may have important diagnostic, prognostic and therapeutic implications. *Methods.* We studied 11 patients with AML at diagnosis (7 males, 3 females, median age 53.5 years, range 19-79) and 6 healthy donors. Five out of the 11 patients were analyzed also at disease relapse. Immunophenotyping of immature cell compartment (CD34+/CD38- cells) was carried out according to a six-color panel of antibodies (Table 1). The samples were acquired by using FACSCanto cytometer (BD Biosciences) and analyzed by FlowJo software (TreeStar). *Results.* The comparative study between healthy donors and AML patients showed significant differences in the expression of some antigens: CD90, CD133 and CD117 were down-modulated in AML patients, as compared to healthy donors. On the other hand, CD123 and CD45RA were upregulated in AML patients. These phenotypic abnormalities were stable at the relapse, as shown by the comparative study performed in 5 relapsed patients. *Conclusions.* The identification of leukemia-associated phenotype in the CD34+/CD38- population can discriminate LSCs from normal HSCs. In addition, this approach may be useful to further strenghten the role of minimal residual disease (MRD) monitoring in AML patients. Consequently, further prospective studies are in progress to demonstrate the clinical relevance of LSC quantification in AML patients.

Table 1.

Samples	FITC	PE	PerCP	PE-Cy7	APC	Apc-h7
01	HLA-DR	CD133	CD38	CD34	CD117	CD45
02	CD25	CD90	CD38	CD34	CD33	CD45
03	CD56	CD123	CD38	CD34	CD7	CD45
04	CD45RA	CD45RO	CD38	CD34	/	CD45
05	mIgG1	mIgG1	CD38	CD34	mIgG1	CD45

PO-103

TARGETING THE LEUKEMIA CELL METABOLISM BY THE CARNITINEPALMITOYLTRANSFERASE1A (CPT1A) INHIBITION: FUNCTIONAL PRE-CLINICAL EFFECTS IN ACUTE MYELOID LEUKEMIAS.

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Introduction. Cancer cells are characterized by perturbations of their metabolic processes, such as the acquisition of a "proglycolitic phenotype" (Warburg effect) and/or an enhanced glutamine catabolism. A novel class of drugs, aimed at targeting the metabolic pathways, are nowadays under investigation. Since the CarnitinePalmitoylTransferase1a (CPT1a) is a protein that catalyzes the first step of fatty acid oxidation by loading long chain fatty acyl groups onto carnitine, transporting them through the mitochondrial membrane, we aimed in this study at evaluating the anti-leukemia effect of the CPT1a inhibition. Particularly, we evaluated the activity of two CPT1a-inhibitors, the well known Etomoxir and the novel ST1326 (kindly provided by Sigma-Tau), on the proliferation and apoptosis of Acute Myeloid Leukemia (AML) leukemia cell lines and in primary cells obtained from AML patients. *Methods.* The cytotoxic effects of ST1326 on AML cell lines (HL-60, HL-60/MX2, U937, K562) and on primary AML were evaluated by MTT test. The drug concentration inducing 50% cell killing (IC50) was calculated from the dose-response curve. The expression of CPT1 in AML cell lines was evaluated by western blot analysis. Flow cytometry Acridine-Orange technique and AnnexinV binding assay were used to examine cell cycle changes and apoptosis. *Results.* The CPT1a expression was preliminary demonstrated in the AML cell lines. Subsequently, we evaluated the activity of ST1326 on AML models, demonstrating at increasing concentration of ST1326, a dose- and time-dependent cell growth arrest, caused by mitochondrial damage and apoptosis induction. The HL-60 cell line, following 72 hours of ST1326 exposure, showed an increase of the subG1 peak from a baseline value of 9.9% to 22.3%, 49.6%, 58.1% and 80.9% at 1, 5, 10 and 50 M, respectively. Similarly, the U937 and HL60/MX2 proved to be highly sensitive to ST1326 (IC50: 8.2 and 8.8 M), while the K562 cell line was resistant (IC50 n.d.). ST1326 resulted significantly more effective compared to Etomoxir (subG1 peak in HL60 at 72 hours remained unchanged: 10.5% at 50 M Etomoxir). The activity of ST1326 was further determined on 12 primary AML samples and a pro-apoptotic activity of ST1326 was observed in all AML samples: AnnexinV positive cells significantly increased at 72-96 hours from 23.27%±13.63 (control) to 36.59%±19.97 (P=0.23), 40.51%±18.66 (P=0.0074), 43.43%±19.81 (P=0.0071) and 75.30%±11.52 (P=0.00018) in the presence of 5, 10, 20 and 50 M of ST1326, respectively. *Conclusions.* ST1326 shows high *in vitro* pro-apoptotic activity on AML models and on primary cells, prompting further studies by molecular inhibition of metabolic pathways in leukemia treatment. Studies are ongoing to evaluate the expression of CPT1a, according to the AML clinical and biological characterization, and to define mechanisms underlying this activity.

PO-104

PATIENT SPECIFIC Q-PCR IDENTIFIES FLT3-ITD SUBCLONES IN FLT3 WILD TYPE AML PATIENTS UNDERGOING RELAPSE WITH FLT3-ITD.

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Introduction. FLT3-ITD mutations are among the most frequent molecular aberrations in AML and they are known to predict poor clinical outcome. Recently A. Nazha et al. (Haematologica 2012) have shown that 4% of patients without FLT3-ITD at diagnosis, who initially achieved complete remission, relapsed subsequently with FLT3-ITD positive AML. However, it is unclear whether the FLT3-ITD was acquired at relapse or if the mutation was present at the subclonal level already at time of first diagnosis. We developed in this study an effective real time quantitative-PCR (RQ-PCR) assay to implement FLT3-ITD detection in individual AML patients who relapsed with this aberration. *Methods.* Four AML patients with wild type FLT3 by routine PCR at diagnosis who relapsed with FLT3-ITD were selected for this study. Leukemic samples collected at relapse were used to determine the genomic sequence of individual ITDs, which allowed to design patient specific forward primers for the RQ-PCR assay using a common reverse primer. The copy numbers of FLT3-ITD were normalized against the reference gene ABL-1. Standard curves were established by 5-fold serial dilutions of FLT3-ITD patient specific plasmid. Sensitivity and specificity tests were carried out for each patient with serial dilutions of RNA with water and wild type RNAs from healthy donors. *Results.* The maximum reproducible sensitivity was 10-4 for 3 patients and 10-5 for one patient. The coefficient of the standard curves for the four samples ranged from 0.995 to 0.999 and the slope from 3,666 and 4,097. The RQ-PCR patient specific assay allowed to identify FLT3-ITD in 2 out of the 4 AML patients. Of interest both these two patients relapsed after 4 and 6 months from initial diagnosis. *Conclusions.* Our results indicate that FLT3-ITD positive subclones can be present at diagnosis and undergo clonal evolution after chemotherapy in AML. Due to the aggressive nature of FLT-ITD AML, its early identification is critical for appropriate patient stratification and therapeutic choice.

PO-105

CORRELATION BETWEEN INDOLEAMINE 2,3-DIOXYGENASE (IDO) EXPRESSION, BIOLOGICAL FACTORS AND RESPONSE TO CHEMOTHERAPY IN ADULT ACUTE MYELOID LEUKEMIA

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Introduction. Tryptophan is an essential amino-acid required for protein synthesis and for cell proliferation.Indoleamine 2,3-dioxygenase (IDO) is a heme-containing enzyme that catalyzes the first and rate-limiting step in tryptophan degradation along the kynurenine pathway. Several studies demonstrated that IDO expression is involved in immune tolerance induction during pregnancy, infection, transplantation, autoimmune diseases and neoplasias. IDO is able to inhibit T-cell function and to induce the transformation of T-cells into regulatory T-cells.IDO is functionally active in several neoplastic diseases and its overexpression by tumor cells and by dendritic cells in tumor-draining lymph nodes is known to be associated to bad prognosis. Our and other groups demonstrated IDO expression in a significant proportion of acute myeloid leukemia (AML) patients. Several observations demonstrated that IDO expression increases along with disease progression. The object of the work is to establish a correlation between IDO expression by leukemic cells, risk factors at diagnosis and patients' outcome. *Methods.* Adult AML patients from the Hematology Institute "L. and A. Seràgnoli" in Bologna were analyzed for risk characteristics at diagnosis and for IDO expression by RT-PCR and by Western-Blot analysis in order to detect IDO protein. Patients were stratified according to age at diagnosis, *de novo* or secondary disease (pre-existing myelodysplastic syndrome or radio-chemotherapy), leucocytosis, cytogenetics (on the basis of cytogenetic characteristics patients were divided into low, intermediate and high risk groups) and FLT3 and NPM mutational status. Eventually the correlation between IDO expression, risk factors at diagnosis and patients' outcome was evaluated. *Results.* Thirteen out of the 45 analyzed patients (28,9%) resulted to be IDO-negative, 71,1% being IDO-positive. Positive patients were divided into three different subgroups according to the IDO level: IDO-low expression, IDO-intermediate expression and IDO-high expression patients. No significant differences in the recurrence of prognostic characteristics at diagnosis between the groups considered were experienced, even if IDO-negative and IDO-low expression patients showed a higher median age at diagnosis than IDO-intermediate and IDO-high expression level.Response to induction chemotherapy regimen was then analyzed among the four groups of patients. Only patients who received cytotoxic chemotherapy were evaluated for response.Intriguingly, it has been found that refractory patients were 71,4% among patients who express IDO at high level and 23,1% among IDO-negative patients. *Conclusions.* IDO-high expression

patients showed a higher proportion of refractory disease than IDO negative patients. It is possible to suppose a role for IDO in the immunogenic cell death mechanism, though a longer follow-up and larger populations are needed to draw definitive conclusions.

PO-106

CKIT MUTATIONS FREQUENCY DETECTED IN PATIENTS WITH ACUTE MIELOYD LEUKEMIA (AML) CHARACTERIZED BY CORE BINDING FACTOR (CBF) REARRANGEMENTS

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Background. Among AML, 38% of patients with CBF rearrangement display cKit mutations, leading to the constitutive activation of the receptor. In particular, the most common mutations involving ex17 (D816V/Y and N822K) are associated with IL3 independence growth and acceleration of the leukemogenic process. Moreover, cKit missense mutations at codon 816 are described in 10-30% of patients with t(8;21) AML and are associated with a poor prognosis, a lower overall survival and event-free survival. *Methods.* We evaluate the presence of cKit ex17 mutations in our cohort of 89 patients with AML at diagnosis [36 currying Normal Karyotype (NK); 31 currying inv(16) and 22 currying t(8;21)] and 32 patients with mastocytosis. In particular, we carried out ARMS-PCRs to specifically amplify D816Y/V and N822K mutations and PCR products were analyzed by DHPLC and sequencing. *Results.* We detected the described cKit mutations in 8% NK AML patients, 43% patients with inv(16), 43% patients with t(8;21) and 53% patients with mastocytosis. In particular, the D816V mutation was detected in 9% (5/53) of patients with CBF AML and in 5% (2/36) of NK AML patients. The D816Y mutation was detected only in t(8;21) and inv(16) AML sub-groups at a similar frequency (14% and 13%, respectively). In contrast, the N822K substitution was more frequent in patients with inv(16) (23%) than NK AML (3%) or t(8;21) (14%). Mastocytosis patients were characterized by D816V/Y mutations in 41% and N822K mutation in 12,5% of the cases. Moreover, our research allowed us to find out a higher frequency of S715 deletion in our AML cohort (respectively 55% (29/53) of CBF AML and 53% (19/36) of NK AML), as well as in 34% of mastocytosis and in 40% of healthy subjects. These data strongly suggest that further studies are required to better understand the functional role of S715 deletion, since this alteration was found also in GIST. Clinical data of CBF-AML patients showed that D816V/Y and N822K correlated with disease related death or no reduction of MRD [80% (4/5) D816V, 57% (4/7) D816Y, 40% (4/10)N822K]. Notably, among t(8;21) AML patients, two out of 22 patients showed both N822K and D816V mutations and did not present any reduction of MRD. One patient achieving MRD reduction in four months, showed a novel cKIT missense substitution F842S (not yet reported in the website of Mutations catalogue of Sanger Institute http://www.sanger.ac.uk). *Conclusion.* We demonstrated that a combination of optimized PCR strategies and DHPLC analysis may be efficiently applied for the detection of cKit mutation in AML patients and could be useful to address selected patients to a synergic approach of targeted drugs (such as Tyrosine Kynase Inhibitors) in combination and conventional chemotherapy.

PO-107

EARLY REDUCTION OF BAALC GENE EXPRESSION DURING INDUCTION CHEMOTHERAPY PREDICTS FOR OUTCOME IN ACUTE MYELOID LEUKEMIA

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Introduction. Since the response to induction treatment of patients with AML is variable, it would be ideal to customize the intensity of treatment to each individual patient, if reliable predictors of response were available. Age, cytogenetics and secondary versus *de novo* disease are the most important factors predicting CR. These factors provide a pre-treatment stratification in risk groups with different probabilities to obtain CR but they are not sufficient to predict the individual response to the first course. Additional stratification could prevent over-treatment and under-treatment by discriminating patients who would benefit from more aggressive procedures, including allogeneic stem cell transplantation. Moreover, given that in most cases induction therapy must be initiated as soon as possible, to collect the necessary information in time to allow stratification at diagnosis is not generally possible. We have previously shown that early peripheral blast clearance (PBC), as assessed by flow cytometry and by WT1 copy reduction, well correlated with CR achievement and outcome in AML. BAALC (Brain And Acute Leukemia, Cytoplasmic) gene is physiologically expressed in CD34+ cells but not in mature bone marrow or blood mononuclear cells and encodes a protein with unknown function. High levels of BAALC expression have been reported in AML, ALL, and CML in blast crisis, and are predictive of adverse clinical outcome in AML patients with normal karyotype. *Methods.* We exploited the kinetics of BAALC transcript to estimate PBC during the first days of standard induction therapy ("3 + 7") in 44 adult patients with AML. Quantification of BAALC gene expression by real time quantitative PCR in peripheral blood on day 1 and 5 of treatment was carried out. The results were expressed as BAALC copy number per 10,000 ABL copies. BAALC ratio was defined as the ratio of copy number measured on day 1 (immediately before starting therapy) and on day 5 (the fifth day after start of treatment, immediately before cytarabine infusion). *Results.* The median BAALC ratio in the overall cohort was 3.32 (range 0.19-2.23). The median BAALC ratio was greater in patients attaining CR as compared to non responders (4.25 vs 0.96, respectively; P=0.004). Furthermore, DFS (Figure 1A) and OS (Figure 1B) were significantly longer in patients displaying a BAALC ratio > 3.32 than in patients with BAALC ratio ≤3.32 (P=0.011, and P<0.034, respectively). *Conclusions.* These data confirmed that PBC is an early predictor of outcome. Its assessment by BAALC kinetics provided accurate stratification of patients since the first days of therapy; as such it deserves potential implications for the management of AML, specifically in order to customize treatment since the outset.

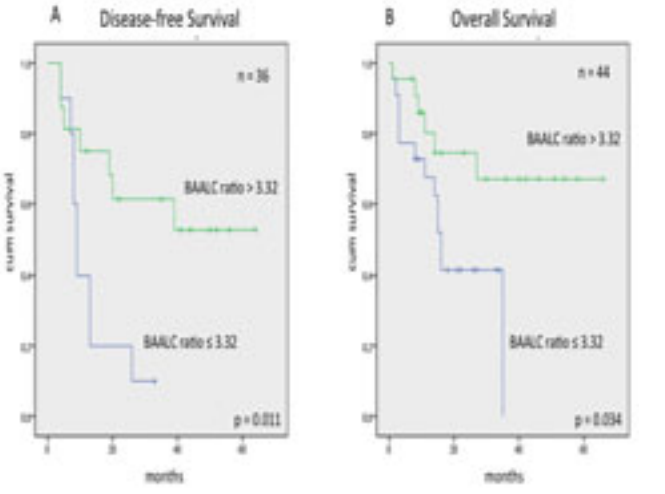

Figure 1.

PO-108

C-KIT MUTATIONS IN CORE BINDING FACTOR ACUTE MYELOID LEUKEMIA

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Introduction. Core Binding Factor (CBF) Acute Myeloid Leukaemia (AML) is a common subtype of AML, defined by the presence of RUNX1-RUNX1T1 [t(8:21)] or CBFB-MYH11 [inv(16)] fusion transcript. Although patients with CBF AML have a more favorable prognosis, there remains a significant failure rate and the long-term disease-free survival is only approximately 60%. Previous studies have demonstrated the presence of activating c-KIT mutations in CBF patients and its association with significantly higher incidence of relapse and lower survival. *Methods.* We retrospectively analysed bone marrow sample of 23

cases with *de novo* CBF AML representing the 7% of newly diagnosed adult AML at AOU S. Giovanni Battista Hospital from 2000 to 2011. Among the total 23 patients under investigation, 14 (60.8%) had inv(16) and 9 (39.2%) had t(8;21). We assessed c-KIT mutations in exons 8, 9, 10, 11, 13, 14 and 17 by PCR amplification in combination with direct sequencing. All patients received standard induction chemotherapy consisting of cytarabine and daunorubicin (ICE). *Results.* c-KIT mutations were found in 7/23 (30.4%) patients. In particular mutation in c-KIT gene were found in 3/9 (33.3%) patients with t(8;21) and in 4/14 (28.6%) patients with inv(16). We reported M541L mutation (exon10) in 3 samples and D816V or D816H or D816Y (exon17) in 4 cases. Moreover we found two SNPs [K546K, I798I)] in 6 AML samples. Consistent with previous studies, the incidence of FLT3ITD, FLT3D835 and NPM1 mutations was low in CBF AML analysed. Among patients there were no significant differences in c-KIT mutation frequencies between cases with sole inv(16) or t(8;21) and cases with additional cytogenetic aberrations. There were no differences in the distribution of sex, age, complete blood cell count, peripheral blood and bone marrow blast percentage at diagnosis according to the c-KIT mutation status. By contrast, a significant difference was found in LDH values between mutated and unmutated patients (P=0.016). In additional analysis of the clinical outcome data with respect to the mutational status of c-KIT there were no significant differences in CR rates. The 10-year overall survival rates were also similar in both groups (63.4% at 136 months vs 53.8% at 108 months), while the survival rates were significantly lower (P=0.003) in all patients with AML compared with the CBF group (28.5% vs 58% at 143 months) as already firmly established. *Conclusions.* In this study we analysed the incidence and the prognostic impact of c-KIT mutations in 23 CBF AML patients. Our results demonstrated an overall incidence of c-KIT mutation of 30.4%, a frequency similar to that previously reported in adult CBF patients. Moreover, we didn't found a prognostic value to stratify patients according to c-KIT mutational status. The discrepancy of our results with other studies might be related to the relatively small number of patients analysed.

PO-109

ULTRA RAPID MOLECULAR DIAGNOSIS OF ACUTE PROMYELOCYTIC LEUKEMIA BY CLOSE-TUBE RETRO TRANSCRIPTION LOOP MEDIATED AMPLIFICATION (RT-LAMP) REACTION

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Introduction. Acute Promyelocytic Leukemia (APL) represents an hematologic urgency. A prompt diagnosis is therefore considered life-saving since patients can benefit from highly effective treatment with retinoids and arsenic trioxide, countering the risk of severe coagulopathies. The genetic diagnosis of APL is based on the detection of the t(15;17) cytogenetic translocation and/or the molecular amplification of one of the PML-RARa fusion transcripts (bcr1, bcr2 or bcr3). *Methods.* we have developed a novel non-PCR method to amplify in 30 minutes the PML-RARa fusion transcripts directly on patient RNA. Two multiplex RT-LAMP assays can be performed to detect, respectively, the most frequent PML-RARa transcripts (bcr1 and bcr3) and the less frequent bcr2 transcript. Beside the targets, both the assays detect the GUSb housekeeping RNA, as internal reaction control, to decrease the risk of false negative results due to poor quality of RNA, wrong reaction conditions or presence of inhibitors. The RT-LAMP reaction is a single step procedure, thanks to simultaneous target retro-transcription, amplification and signal detection by real-time monitoring of fluorescence, thus avoiding the need for DNA production and gel separation. *Results.* the performances of the triplex (bcr1-bcr3-GUSb) and duplex (bcr2-GUSb) RT-LAMP assays have been evaluated on RNA extracted from NB-4 cell line (positive for bcr1) and on RNA obtained from patients presenting the PML-RARa bcr1 or bcr3 transcripts. Test sensitivity has been established on serial dilution of mutated RNA into wild type RNA (from HL-60 cell line): the 10-3 dilutions (30 replicates each) have been always detected within 30 minutes. The level of specificity is 100%, since no false positive results have been obtained on 300 replicates of wild type cell lines. The reactions have been validated on 30 clinical samples from patients affected by APL, previously diagnosed by RT-PCR, obtaining fully concordance with the conventional method. *Conclusions.* The RT-LAMP assays developed demonstrated a high sensitivity and specificity in detection of bcr1, bcr2, bcr3 and GUSb internal control RNA. The close-tube, one-step fluorescent format maximizes the reliability of the method by reducing the risk of errors and contamination. The RT-LAMP can meet the need of timely and reliable diagnosis of Acute Promyelocytic Leukemia in clinical practice, representing a life-saving tool for APL patients.

PO-110

NEGATIVE IMPACT OF ABCG2 OVEREXPRESSION IN PATIENTS UNDERGOING ALLOGENEIC TRANSPLANT FOR ACUTE MYELOID LEUKEMIA

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Introduction. Over-expression of MDR-related protein ABCG2 is associated with higher relapse risk in acute myeloid leukemia (AML) patients achieving remission with chemotherapy. However, little is known on the impact of ABCG2 in patients undergoing allogeneic stem cell transplant (SCT). We retrospectively analyzed the effect of ABCG2 expression in 135 patients with AML who underwent allogeneic SCT at our Institution (between 2002 and 2011). *Methods.* Fifty-seven of 135 patients (42%) over-expressed ABCG2, while 78 cases (58%) did not. Median age at SCT was 48 (range: 17-70) years. Sixty eight patients (50%) received grafts from a sibling donor, 67 (50%) from a matched unrelated donors (MUD). In 88 patients (65%) stem cells source was peripheral blood (PB), while bone marrow (BM) stem cells were used in 47 cases. Status at SCT was complete remission (CR) in 91 patients, while 44 patients were transplanted with relapsed or refractory disease. *Results.* Two-years progression-free survival (PFS) from transplant was 55% for the entire population. PFS was not associated with patient's age, donor type, stem cell source or CD34+ quantity. The only factors positively affecting PFS were status at transplant (CR vs active disease, P<0.0001) and ABCG2 status (negative vs positive, P= 0.02). Among 57 ABCG2+ patients, 31 relapsed (53%), compared with 25 relapses in 78 ABCG2- cases (35%). As a consequence, both 1-year and 2-years PFS were higher in the ABCG2- patients (70% and 67%, respectively) than in the ABCG2+ patients (51% and 46%, respectively). The difference in relapse rate between the two cohorts was significant in the 91 patients transplanted in CR (13 relapses in 36 ABCG2+ vs 8 relapses in 55 ABCG2-, P= 0.03) while was similar in the 44 cases who underwent SCT with active leukemia (18/21 in ABCG2+ vs 17/23 in ABCG2-, P= 0.55). *Conclusions.* Our data suggest that overexpression of ABCG2 is associated with a worse outcome in patients undergoing SCT for AML, mainly for a higher risk of relapse, especially in those transplanted in CR. This finding could suggest for a stricter follow-up and prompt intervention (e.g. DLI) in ABCG2+ cases.

PO-111

BAALC OVEREXPRESSION IS ASSOCIATED WITH POOR PROGNOSIS IN ACUTE MYELOID LEUKEMIA PATIENTS ACROSS ALL CYTOGENETIC RISK CATEGORIES

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Introduction. Overexpression of brain and acute leukemia cytoplasmic (BAALC) gene confers poor prognosis in patients with AML with normal cytogenetic. Less defined is its role in AML with cytogenetic abnormalities. We evaluated the role of BAALC overexpression on outcome of adult AML patients, irrespectively of karyotype. *Methods.* We analyzed 210 patients with AML treated at the Division of Hematology of Udine between 2005 and 2011. Median age was 60 years (range: 20-87). Karyotype was available in 186 patients: favorable = 23, intermediate = 109 (normal = 79), unfavorable = 54. Quantitative BAALC expression was determined by real-time PCR and expressed after normalization to ABL; the cut off value was set at 50th percentile. *Results.* BAALC was overexpressed in 89/210 (42%) patients. No association was found between BAALC positivity and sex, age, type of leukemia, WBC count, FLT3-ITD status or CD56 expression. High BAALC expression was asso-

ciated with CD34 positivity (71/89 vs 49/121, P<0.00001). BAALC overexpression was lower in patients with favorable karyotype (4/23, 13%) than in all others cytogenetic groups (67/163, P=0.05). Considering intensity of expression, higher BAALC levels were found in CD34+ (P=0.0004) and in patients with intermediate karyotype, compared to those with favorable or unfavorable abnormalities (P=0.04 and P=0.05, respectively). 191 of 202 patients (8 APL cases were excluded) were evaluable for therapy response. In multivariate analysis, BAALC overexpression had a negative impact on CR achievement (P=0.002), as well as age and CD34+. BAALC did not influence relapse probability, but had a strong impact on OS: 3-years OS was 50% in BAALC- compared to 36% in BAALC+ patients (P=0.001). Karytotype per se did not influence survival probability, but subgrouping patients according to cytogenetic risk and BAALC expression we identified three groups with different prognosis: favorable karyotype + low BAALC (3-years OS 75%), intermediate karyotype irrespective of BAALC and unfavorable karyotype + low BAALC (3-years OS 48%), favorable or unfavorable cytogenetic + high BAALC (3-years OS 34%) (P=0.02). *Conclusions.* BAALC overexpression identified AML patients with poor prognosis in all cytogenetic groups. Though rare, BAALC positivity in patients with favorable or unfavorable karyotype significantly worsened survival.

PO-112

FLUDARABINE-BASED INDUCTION THERAPY DOES NOT OVERCOME THE NEGATIVE IMPACT OF BAALC OVER-EXPRESSION IN ACUTE MYELOID LEUKEMIA PATIENTS

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Introduction. Inclusion of fludarabine in induction therapy overcomes negative effect of MDR over-expression in acute myeloid leukemia (AML) patients, increasing remission rate and survival. Less known is the impact of fludarabine on newer molecular alterations associated to negative prognosis. We evaluated the impact of various molecular features on the outcome of AML patients treated at our Institution with a fludarabine-based induction. *Methods.* We retrospectively analyzed 140 consecutive patients with non-M3 AML receiving fludarabine-based induction and at least one consolidation course with high-dose cytarabine between 2004 and 2011. MDR proteins (ABCB1, ABCG2) expression was evaluated by multiparametric flow cytometry. FLT3-ITD and NPM1 mutations were tested by standard methods, BAALC expression was calculated by real time PCR, with the cut-off value set at 50th percentile. *Results.* Median age was 55 (range: 20-84) years. Karyotype was evaluable in 130 patients: 7 (5%) favorable, 83 (64%) intermediate and 40 (31%) unfavorable. ABCB1 and ABCG2 were over-expressed in 39 (28%) and 60 (43%) cases, respectively. FLT3-ITD and NMP1 mutations were present in 38 (27%) and 36 (26%) patients, while BAALC was over-expressed in 61 (44%). After induction therapy, 101 (72%) patients achieved CR and 39 (28%) were refractory. In multivariate analysis, CR was negatively affected by secondary leukemia (P=0.003) and BAALC over-expression (P=0.035). Thirty-one of 101 (31%) patients relapsed; relapse probability was affected only by ABCG2 over-expression (P=0.03). Four-years overall survival (OS) was 49% (95% CI: 40-60). Lower survival probability was associated with age (P=0.0001), ABCG2+ (P=0.003) and BAALC over-expression (P=0.02), not with FLT3 and NPM1 mutations. However, subdividing patients according to FLT3, NPM1 and BAALC status, we identified three groups with significantly different OS: FLT3-/NPM1+/BAALC± patients showed the best survival, FLT3+/NPM1-/BAALC±, FLT3+/NPM1+/BAALC+ and FLT3-/NPM-/BAALC+ patients had the worst survival, while FLT3-/NPM1-/BAALC- and FLT3+/NPM1+/BAALC- patients displayed intermediate OS (P=0.002). *Conclusions.* BAALC over-expression has a negative impact on CR attainment and OS in AML patients, irrespectively of cytogenetics and, to a lesser extent, FLT3 and NPM1 mutations, except the FLT3+/NPM1- subgroup. Fludarabine overcomes the negative impact of ABCB1+ and FLT3-ITD mutation, but seems to have no impact on BAALC over-expression.

PO-113

ACHIEVEMENT OF COMPLETE REMISSION WITH PONATINIB THERAPY FOR PHILADELPHIA POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA RELAPSED AFTER ALLOGENEIC STEM CELL TRANSPLANTATION: A CASE REPORT

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Introduction. The prognosis of Philadelphia positive Acute Lymphoblastic Leukemia (Ph+ ALL) relapsed after allogeneic stem cell transplantation (allo-SCT) is very poor. Salvage therapy with third generation tyrosine-kinase inhibitor (TKI) Ponatinib is a valid option for patients with BCR-ABL T315I mutation. *Methods.* We report the case of a 62 years old woman with P190 BCR-ABL Ph+ ALL transplanted in complete remission (CR) on 20th October 2010 with a HLA antigen 5/6 matched unrelated donor (MUD). At the time of SCT the patient was in first complete cytogenetic remission and in major molecular response after standard induction/consolidation chemotherapy, including central nervous system (CNS) prophylaxis. The peripheral CD34+ reinfused were 4,8× 10^6/Kg and CD3+ were 228×10^6/Kg. The patient was conditioned with reduced intensity conditioning regimen including Thiotepa and Cyclophosphamide. Graft versus host disease (GVHD) prophylaxis consisted on ATG, Cyclosporine and short course Methotrexate (MTX). Donor stem cells engraftment was observed on day +19. No major complications developed during the aplastic phase. Grade I cutaneous GVHD was documented and resolved without steroid therapy. A bone marrow aspirate at day +30 confirmed the CR with a complete donor chimerism which was maintained until day +210 when a bone marrow relapse and chimerism loss associated with CNS involvement were documented. Donor lymphocytes infusions were not possible because of donor unwillingness. Mutational analysis showed no mutation in the ABL gene. We started TKI therapy with Dasatinib (140 mg/die) and intrathecal chemotherapy including Cytosine Arabinoside, MTX and steroid with achievement of complete remission but the patient relapsed two months later and BCR-ABL transcript analysis showed T315I mutation. We administered conventional chemotherapy (Vincristine and steroid) to control the disease burden while we were waiting for Ponatinib that was available under a compassionate program. The drug was started on 5th March 2012 at the standard dose of 45 mg/day. *Results.* On day +32 of Ponatinib therapy we obtained a complete remission disease with complete cytogenetic response. The molecular disease burden reduced by two Log. Molecular chimerism was 100% donor (Figure 1). We stopped therapy for 4 days because of development of neutropenia (WHO grade IV) and skin rash (WHO grade II) that was treated with low dose of steroid. Ponatinib therapy was restarted at a lower dosage (30 mg/day) without other drug related toxicity. The patient is now under evaluation for CNS radiotherapy in order to prevent CNS recurrence. *Conclusions.* Third generation TKI Ponatinib may be a very effective therapy for Ph+ ALL relapsing after allo-SCT, that are resistant to other TKIs for the presence of T315I mutation. A bigger survey is necessary to address some questions about synergism between donor hematopoiesis and Ponatinib capacity to obtain a deep minimal residual disease clearance.

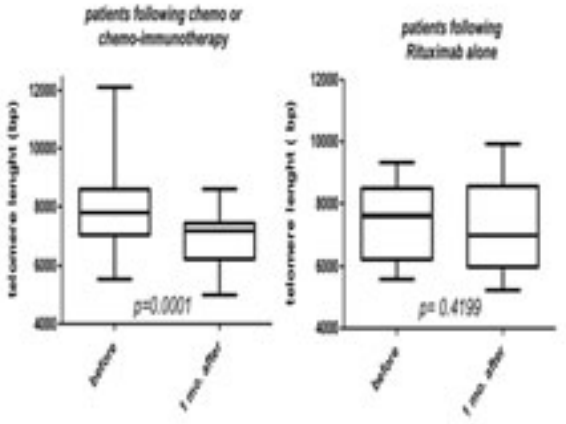

Figure 1. Bone marrow molecular monitoring of minimal residual disease and donor chimerism of a patient with Ph+ ALL treated with TKI at relapse disease after allo-SCT.

PO-114

AGE-SPECIFIC DISTRIBUTION AND SURVIVAL OF CYTOGENETIC SUBGROUPS OF ACUTE MYELOID LEUKEMIA IN ROMAGNA

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Introduction. It is well known that the different cytogenetic subgroups of acute myeloid leukemia (AML) have discrete prognostic significance and show different age-specific frequencies. We tested this fact in a series of patients with AML observed and followed in 2 Hematology Departments in Romagna from 1995 to 2010. *Methods.* We calculated the distribution of the major cytogenetic abnormalities (1), according to (2), into the population of patients divided according to age >/= or <60 years. *Results.* Within a 16 years period, 340 patients with AML were admitted to the Hematology wards of Ravenna and Rimini. 192 were males and 148 females. Their age varied between 20 and 90 tears, media 64 years. As a whole, 34 patients showed a t(8;21), or a inv(16) or a t(15;17) and were included in the favourable group (12%); 74 patients had a complex karyotype or missing/deletion of chromosomes 5 or 7 and were included in the unfavourable group (27%). Other abnormalities and normal karyotypes were observed in 160 patients (112 normal), included in the intermediate group (61%). With a cut-of at 60 years, of the 88 (32%) young patients, 16 (18%) were in the favourable group, 56 (64%) in the intermediate one and 16(18%) in the unfavourable one, while of the 190 old patients, 18 (9%) were in the favourable group, 114 (60%) in the intermediate one and 58 (31%) in the unfavourable one, respectively. Figure 1 shows the survival curves of the patients divided according to the prognostic groups and age. In the group with favourable cytogenetics, old patients had a median survival of 57 months, while 77% of young ones were alive at 5 years. In the intermediate group, old patients had a median survival of 10 mos vs 13 mos for young patients. Finally, in the unfavourable group, old patients had a median survival of 4 mos vs 16 mos for young ones, respectively. *Conclusion.* We confirm previous observations regarding the higher incidence of complex/unfavourable karyotypes in older patients (2). Moreover, in our series, cytogenetics play a strong prognostic role, negatively influenced by age. *Aknowledgements*. Ravenna AIL and Network Ematologico Regione Emilia Romagna (NERER).

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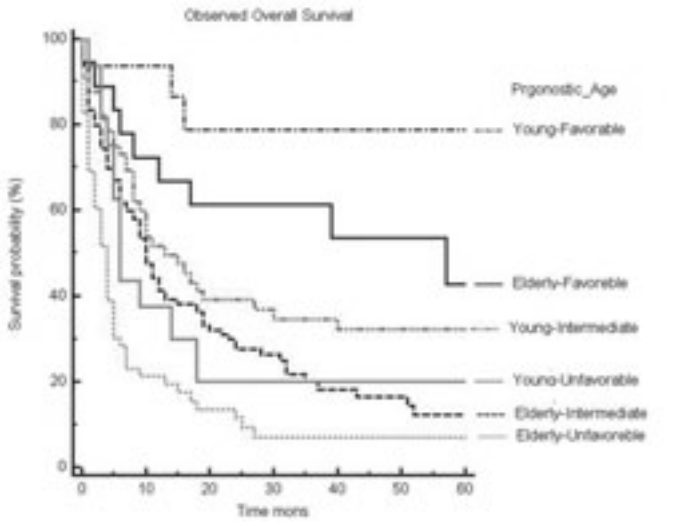

Figure 1. Survival of AML patients according to cytogenetic subgroups.

Chronic Lymphocytic Leukemia

PO-115

THE ENZYME NICOTINAMIDE PHOSPHORIBOSYL-TRANSFERASE (NAMPT) CONTRIBUTES TO CREATING A PROINFLAMMATORY ENVIRONMENT IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction. Tumor transformation is accompanied by an altered metabolic state, with higher needs for NAD, an essential co-factor in the oxidative phosphorylation chain. In leukocytes, NAD is synthesized mainly from nicotinamide through the activity of NAMPT, the first and rate-limiting enzyme in this biosynthesis pathway. NAMPT can also be present extracellularly [eNAMPT, aka pre-B cell colony enhancing factor (PBEF) or visfatin], where it exerts cytokine-like actions. eNAMPT is over-expressed in different tumor models as well as in acute and chronic inflammatory-metabolic diseases. The aim of this work is to test whether eNAMPT plays a role in shaping the leukemic environment, by generating pro-inflammatory conditions that favor tumor expansion. The model selected is chronic lymphocytic leukemia (CLL), a disease characterized by the slowly progressive expansion of mature CD5+ B lymphocytes, dependent on a growth supportive environment for progression. A further reason for selecting this model is that CD38, the main mammalian NADase, is a negative prognostic marker for CLL patients, suggesting that modulation of the extracellular NAD/nicotinamide balance is critical in determining a more aggressive phenotype.

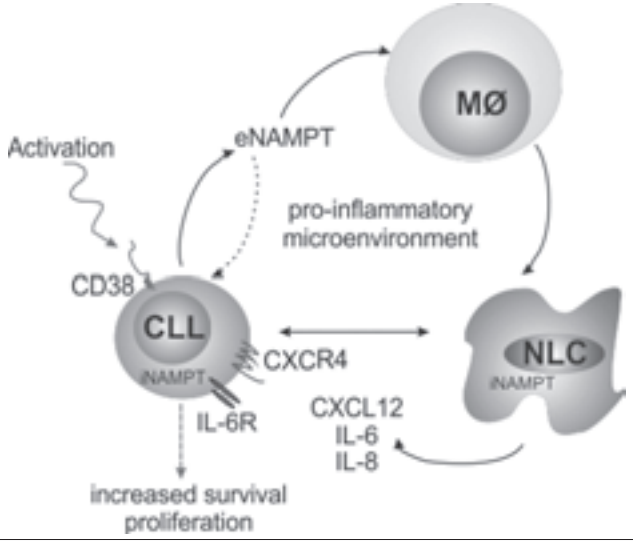

Figure 1.

Methods. CLL or normal B lymphocytes were purified by negative selection. NAMPT expression was determined by western blot and qRT-PCR. eNAMPT was quantified by ELISA (Adipogen). Cell morphology was studied using Giemsa staining of cytospin preparations, cytofluorimetric analyses and confocal microscopy. *Results.* The first finding of the work is that CLL lymphocytes express higher levels of NAMPT mRNA when compared to normal B lymphocytes obtained from age- and sex-matched donors. Plasma eNAMPT levels are also significantly higher in CLL patients when compared to controls (7.1 ng/mL vs 2.3 ng/mL, P=0.002). Activation of purified CLL cells is followed by eNAMPT secretion, indicating that it is the leukemic component that actively releases eNAMPT. Treatment of PBMC, but not of purified CLL lymphocytes, for 5 days with recombinant NAMPT is followed by secretion of significant amounts of IL-6 and IL-8. Furthermore, exposure of PBMC cultures to eNAMPT induces the appearance of a population of adherent cells, dis-

playing intracellular vacuoles and granules compatible with macrophage differentiation (CD14+/CD11b+/CD11c+). Lastly, long-term exposure (2 weeks) to eNAMPT enhances the formation of nurse-like cells (NCLs), large, round and adherent myeloid cells, considered essential components of the CLL microenvironment. Treatment of this cell population with eNAMPT triggers phosphorylation of Erk1/2, Akt (Ser473) and nuclear translocation of the NF-kB component p65. *Conclusions.* These data support the hypothesis that eNAMPT contributes to the formation of a pro-inflammatory environment, in which activated CLL cells secrete eNAMPT, driving recruitment and differentiation of a myeloid population, with features typical of tumor-associated macrophages.

PO-116

BIOLOGICAL CHARACTERISTICS IN 340 PATIENTS AFFECTED BY CHRONIC LYMPHOCYTIC LEUKEMIA DIAGNOSED IN A SINGLE CENTRE OF HAEMATOLOGY DURING THE LAST 12 YEARS

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Introduction. Chronic Lymphocytic Leukemia (B-CLL) is the most common lymphoprolipherative disorder of the elderly population in Western countries. Biological characteristics in B-CLL are useful for prognosis and management of the disease. As they could change during the time, they should be studied at the onset of the disease and at the time of progression. This retrospective study has the purpose to analyse biological characteristics of B-CLL patients (pts), diagnosed and followed at a single centre of Haematology. *Methods.* From our database, consisting of 447 diagnosis during the last 12 years, we excluded 107 patients previously treated in other centre or referred to our centre for consultancy. Thus, we selected 340 patients affected by B-CLL, diagnosed from January 1999 to December 2010 and followed until March 2012. At the diagnosis, we studied the main biological features of each new patient as IgVH mutational status, chromosomal aberrations and immnunophenotype comprehensive of ZAP-70, CD38 and CD49d. *Results.* We recorded a mean of 28 B-CLL diagnosis per year (range 14-43). The analysis of the IgVH mutational status, available in 265 (78%) pts, displayed 156 pts (58.9%) mutated and 109 pts (41.1%) unmutated. The analysis of chromosomal aberrations, conducted in 297 pts (87%) by fluorescence in situ hybridization (FISH), demonstrated abnormalities in 48.1% of the pts (143 pts). We found del(13q14) in 77 pts (25.9% of the investigated pts), +12 in 45 pts (15.1%), del(11q22) in 26 pts (8.7%) and del(17p13) in 18 pts (6.1%). Cytofluorimetric analysis showed positivity of ZAP-70 in 116 pts (37.7%; data available for 308 pts), CD38 in 76 pts (24.1%; data available for 315 pts) and CD49d in 58 pts (42.6%; data available for 136 pts). Then we correlated the main biological characteristics of the disease with the outcome (Table 1).

Table 1. Correlations between main biological characteristics and outcome

	Mutated IgVH	Unmutated IgVH	Standard risk chromosomal aberrations (normal karyotype, +12, del 13q)	High risk chromosomal aberrations (del 11q, del 17p)
N° pts	156	109	254	43
N° progressions (%)	67/156 (43%)	78/109 (72%)	124/254 (49%)	39/43 (91%)
TTP (median time)	n.r. (53 months)	17 months	n.r. (45 months)	24 months
N° treatments (%)	47/156 (30%)	77/109 (71%)	99/254 (39%)	39/43 (91%)
TTT (median time)	n.r. (61 months)	19 months	n.r. (51 months)	24 months
N° deaths or lost at follow-up (%)	31/156 (20%)	46/109 (42%)	61/254 (24%)	20/43 (46%)
OS (median time)	n.r. (78 months)	n.r. (63 months)	n.r. (70 months)	n.r. (75 months)

TTP: Time To Progression; TTT: Time To Treatment; OS: Overall Survival; n.r.: not reached.

Unmutated IgVH pts underwent to progressive disease and started treatment after a median time of 17 and 19 months respectively. Mutated IgVH pts did not show progressive disease or require treatment after a median time of 53 and 61 months respectively. At present 43% and 30% of mutated pts experienced progression or treatment respectively. On the basis of FISH analysis, the median time of progression and treatment in "high risk" pts (del 11q and del 17p) was 24 months in both, while "standard risk" pts (normal karyotype, + 12, del 13q) did not achieve median progression and treatment at 45 and 51 months respectively. Median overall survival was not reached for mutated/unmutated IgVH groups and for high and standard risk FISH groups. *Conclusions.* This wide survey is the first reported in the literature, comprehensive of biological and clinical features from a single centre experience. Our population is representative of the biological characteristics of the B-CLL.

PO-117

LEUKEMIC CELLS FROM B-CLL PATIENTS DISPLAY HETEROGENEOUS RESPONSE TO THE ANTI-APOPTOTIC EFFECT OF MESENCHYMAL STROMAL CELLS

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Introduction. The malignant behaviour of chronic lymphocytic leukemia (CLL) cells cannot be exclusively ascribed to their intrinsic features, but also to factors originating from the surrounding microenvironment. In the active sites of the disease, such as the bone marrow (BM) and secondary lymphatic tissues, CLL B-cells engage complex, yet incompletely defined, cellular and molecular interactions with stromal cells, affecting their survival and growth and conferring drug-resistance. Mesenchymal Stromal Cells (MSCs) represent the dominant population in CLL-marrow stroma, differently from other B-cell lymphomas and healthy subjects, and are involved in supporting leukemic B-cell survival. Despite the remarkable phenotypic homogeneity of neoplastic B cells, CLL is characterized by a variable clinical outcome, suggesting that CLL clones should display heterogeneous features also in responding to environmental pro-survival signals. In this study we investigated whether different CLL clones show a different susceptibility to spontaneous apoptosis when co-cultured in presence of MSCs recovered from B-CLL patients. *Methods.* MSCs isolated from the BM of 47 B-CLL patients were expanded ex vivo and characterized through flow cytometry analysis and differentiation cultures (adipocytes and osteocytes). Freshly isolated CLL peripheral blood B-cells were co-cultured with autologous and allogenic CLL-MSCs or with the stromal cell line HS5, used as control. Apoptosis was measured by Annexin V test and western blotting analysis. *Results.* The survival of neoplastic cells ranged from 13.3% (±13.2%) when leukemic cells were cultured in medium alone, to 58.5% (±17.2%) when cultured in presence of CLL-MSCs (P<0.01). The presence of the 85KDa cleaved PARP fragment in all CLL B-cells cultured in medium alone confirmed that they underwent spontaneous apoptosis, as compared to CLL cells co-cultured with different CLL-MSCs. Interestingly, we observed that even if different CLL-MSCs exert the same pro-survival effect on leukemic cells, different CLL clones, co-cultured in the presence of the same CLL-MSC line, exhibit heterogeneous levels of apoptosis (Figure 1A). On the contrary, CLL B-cells co-cultured in presence of the stromal cell line HS5 displayed the same apoptotic rate (Figure 1B).

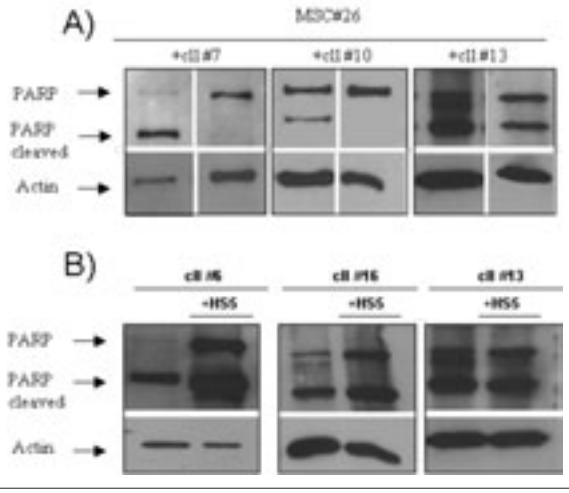

Figure 1.

Conclusions. CLL tumor cells are more prone to apoptosis when cultured ex vivo, because they lack pro-survival signals provided *in vivo*. MSCs isolated from different CLL patients displayed their anti-apoptotic effects on CLL cells in a similar way. At the same time, each CLL-clone shows a peculiar response to signals coming from the marrow microenvironment, suggesting that CLL clone could be more or less responsive to microenvironment protection. This finding open the way to tailored therapies which powerfully target the cross-talk with marrow elements (i.e. lenalidomide), especially on patients carrying a clone more sensitive to anti-apoptotic signals from microenvironment.

PO-118

EARLY EXTRAMEDULLARY MYELOMA RELAPSE (EMR) IN THE TARGETED THERAPIES ERA: ANAPLASTIC EVOLUTION WITH BULKY MULTIFOCAL PRESENTATION

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Introduction. EMR in Multiple Myeloma (MM) is a rare occurrence ranging from 6% to 20% during the course of the disease leading to shorter overall survival and progression free survival. In order to describe the EMR features we screened the myeloma registry of our institution. *Methods.* From January 2003 to January 2012 among 200 MM pts 6 (3%) EMR were recorded. All were males, median age 59 y (range 49-67). At diagnosis, 3 had IgA, 2 IgG and 1light chain MM, respectively; 5 were stage III D-S, renal impairment was found in 3 cases, ISS 3 in 2 pts only. Cytogenetics was available in 5 cases; chromosomal abnormalities were detected in 4 cases: 13 q- (2), hyperdiploid karyotype(1) and complex karyotype (1). As 1st line therapy, 3 pts received VAD, 3 bortezomib schedules, respectively; 5 of them underwent ASCT and subsequently bortezomib or thalidomide maintenance. *Results.* Median time between MM diagnosis and EMR was 34 mos (12-41). In all cases, EMR presented as bulky (>5 cm) disease, with a plurifocal spread including leptomeningeal+sphenoid, bone+skin, multiple muscular involvement, liver+peritoneal serus+ colon, mediastinum, larynx. Bone marrow infiltration was absent in 3 pts, while 2 mg, LDH and VES were high in 3, 4 and 6 pts, respectively. EMR treatment included bortezomib+ dexametasone +/- cyclophosphamide (2), lenalidomide +dexametasone (2), lenalidomide+bendamustine (1), liposomal doxorubicin (1). Only 1 pt achieved response wich lasts for 27 mos. Median overall survival from EMR was 8 mos (2-33). *Conclusions.* Like other experiences, also in our series EMR occurrence resulted low (<10%), but with homogeneous clinical characteristics: anaplastic evolution, bulky expression, multifocal spread, absent or reduced bone marrow infiltration, cytogenetic abnormalities. To date, it is not possible to identify, at MM diagnosis, which pts will develop a EMR, since current prognostic index are not predictive of this event. In the novel agents era, the EMR occurrence may be correlated to a longer survival. In our series EMR occurred in 4 pts after a prolonged (>12 mos) maintenance with bortezomib or thalidomide/lenalidomide post ASCT. To date, the biologic mechanisms with which the anaplastic clone may be selected out of bone marrow are not completed clear. Futhermore decreased adhesion molecule expression and down-regulation of chemokine receptors have been recorded in EMR plasmacells, which escape the immune surveillance. It is very likely that absence of marrow microenvironment represents the main cause of failure of EMR salvage therapy. Further studies are needed to evaluate future therapeutic approaches in this setting.

PO-119

MERKEL-CELL POLYOMAVIRUS AND B-CELL CHRONIC LYMPHOCYTIC LEUKEMIALEUCEMIA LINFATICA CRONICA E MERKEL CELL VIRUS

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Introduction. Some authors described a possible association between CLL and Merkel cell polyomavirus (MCPyV) the pathogenetic agent of the Merkel-cell carcinoma, a rare and aggressive carcinoma of the skin. This association suggests the possibility that MCPyV may play a role in the pathogenesis of CLL as well as in the progression of the disease. Given several contradictory reports on the role of MCPyV in CLL, the presence of this virus as well of the other polyomaviruses JCV, BKV and of other lymphotropic viruses i.e. EBV and SV40 was investigated in highly purified malignant cells of a large number of CLL patients in different stages of the disease. *Methods.* Blood samples were obtained from 66 patients fulfilling diagnostic criteria for CLL. In each case the IGHV gene mutational status was previously assessed using standard methods and CD38 and ZAP-70 expressions were determined by flow cytometry. Interphase FISH was performed on nuclei preparations of PBMC and each case was investigated for 13q deletion, 11q deletion, 17p deletion or presence of trisomy 12. The samples were processed immediately after blood withdrawl and processed by Ficoll-Hypaque density gradient resulting in purity of > 98% of CD19+/CD5+ CLL cells as assessed by expression analysis on flow cytometry. DNA was extracted by using a commercial and stored at -80 C° until the time of analysis. A multiple Q-PCR assay was run to amplify both Polyomavirus JCV, BKV and MCPyV. Real time Q-PCR for MCPyV Tag sequences, viral loads and the cellular beta-globin gene (as reference gene) were performed in each sample. Infection of EBV was evaluated by specific quantitative real time PCR (Q-PCR) assay using a commercially available molecular kit (Nanogen, Italy). The lowest limit of EBV detection assays was 100 copies/reaction and for Polyomavirus 10 copies/reaction. Q-PCR assays were run on the AB PRISM 7900 Sequence detection System (Applied Biosystems, Milan, Italy). Multiple negative controls (containing water instead of DNA templates), and positive controls (containing plasmid with the entire viral genome) were included with each assay batch. *Results.* Among the 66 patients one case only (1.5%) was positive for MCPyV, while no one was positive for SV40, JCV and BKV. Five cases (7.6%) were positive for EBV replication in PBMC. The EBV positivity was independent from age, IGVH mutational status or from ZAP70 and CD38 expression or from the presence of 17p deletion, while correlates with previous treatments. The patient positive for MCPyV was affected by Merkel Cell skin carcinoma. This patient at diagnosis was negative for both MCPyV and EBV and underwent 4 therapy lines before positivity. *Conclusions.* The low frequency of detection of MCPyV DNA in B-CLL patient samples, points against a direct involvement of MCPyV in CLL pathogenesis. Likely, the immune-depression of CLL, exacerbated by chemio- and immune-therapies, results in reactivation of both EBV and MCPyV.

PO-120

MINIMAL RESIDUAL DISEASE LEVELS DETERMINED BY FLOW CYTOMETRY PREDICT RESPONSE DURATION AND OVERALL SURVIVAL IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Today in CLL chemotherapy is combined with monoclonal antibodies (MoAbs) and this approach produces more complete remissions (CR) and longer response duration (RD), remaining often a minimal residual disease (MRD) detectable only by flow cytometry (Keating, 2005). In addition, consolidation/maintenance therapy with MoAbs might provide a further clinical outcome benefit in CLL, as well as already achieved in other indolent non-Hodgkin lymphomas. From 1998 to 2010, we treated in first line 146 CLL symptomatic patients (pts), median age 63 years, with six monthly courses of intravenous (25 mg/sqm)

or oral fludarabine (30-40 mg/sqm) and then, after a median time of 30 days, with four weekly doses (375 mg/sqm) of rituximab (rtx). Before treatment, 15 pts had a modified low Rai stage, 127 an intermediate stage and only 4 a high stage. We defined as high risk pts having at least two of these markers: unmutated IgVH, CD38>30%, ZAP-70>20%, intermediate/ poor cytogenetics (trisomy 12 or del11q or del17p). Sixty-three pts (43.1%) belonged to the high risk subset. MRD study was performed by multicolor flow cytometry (FACSCalibur, BDIS) and the threshold of positivity was set at >1% CD19+CD5+CD79b+/- bone marrow (BM) CLL cells. This immunological evaluation was carried out within 8 weeks from the end of induction treatment. Based on NCI criteria, 111/146 (76%) pts achieved CR, 28/146 (19%) a partial remission (PR) and 7/145 (5%) no response or progression. Phenotypic CR (CD19+CD5+ CD79b+/- BM cells <1%) was achieved in 84/145 (57%) CLL pts. MRD positivity was significantly correlated with a high risk biologic profile (38/61; 62%, P=0.0001) Interestingly, MRD+ pts showed significant shorter response duration (18% vs 60% at 8 years; P=0.00009) and shorter overall survival (OS) in comparison with MRD- pts (25% vs 73% at 16 years, P=0.0001). Fifty-nine pts (43%) either in CR with B-CLL BM cells >1% (MRD+, n=16 pts) or in CR MRD negative, but developing MRD positivity within 2 years after induction (n=25 pts) or in PR (n=18 pts), underwent consolidation/maintenance therapy with four monthly cycles of rtx at 375 mg/sqm followed by twelve monthly doses of rtx at 150 mg/sqm. The median follow-up duration was 63 months. Noteworthy, both persistently MRD negative pts (n=56) and pts undergoing consolidation/maintenance therapy (n=59) showed a longer RD vs MRD+ not consolidated pts (n=23) [75% vs 58% vs 0% at 5 years; P<0.0001, Figure]. Equally, OS was shorter in MRD+ not consolidated pts in comparison with the other two subsets (0% vs 63% vs 78% at 16 years; P=0.03, Figure). Noteworthy, within the high risk subset (n=63), pts in persistent phenotypic CR (n=18) and consolidated pts (n=23) showed a longer RD (90% vs 66% vs 8% at 2.7 years, P=0.00024) vs MRD+ not consolidated pts (n=15). Therefore, in our experience, MRD determination by flow cytometry represented a key factor for establishing a consolidation/maintenance treatment allowing us to prolong RD and OS in CLL.

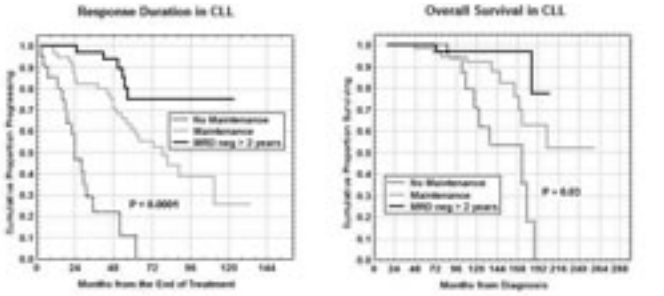

Figure 1.

PO-121

CLINICAL MONOCLONAL B LYMPHOCYTOSIS IS CHARACTERIZED BY A BIOLOGICAL LOW RISK PROFILE PREDICTIVE OF A FAVORABLE CLINICAL COURSE

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Monoclonal B-cell lymphocytosis (MBL) is an asymptomatic monoclonal expansion of <5.0×10^9/L CLL (chronic lymphocytic leukemia)-phenotype B-cells. CLL-like MBL, defined as clinical MBL (cMBL), demonstrated a substantial risk of leukemic progression to symptomatic disease requiring treatment that can be predicted by biological prognosticators (Rawstron, 2008). Based on this background, the aims of our study were: (i) to define a biological profile and clinical features of cMBL in comparison with Rai 0 stage; (ii) to determine treatment free survival (TFS) and overall survival (OS) of cMBL and Rai 0; and finally (iiii) to validate cMBL as an independent prognostic factor. For this purpose, we investigated 583 patients, median age 66 years, 322 males and 261 females. One-hundred sixteen cases were defined as cMBL and 467 cases as CLL (Rai 0: 135; I-II: 320; III-IV: 12). The database was updated for the analysis in December 2011. The median follow up was 6.8 years. Among cMBL, median absolute lymphocyte count was 7.3×10^9/L and median CLL-phenotype lymphocyte count was 3.5×10^9/L. Overall, 54/116 cMBL (46.5%) progressed to overt CLL and median time to progression to CLL was 43.5 months. The risk of progression to CLL was 8.3% per year in the first 6 years and decreased thereafter to 1.25%. Moreover, cMBL with CLL-lymphocytes <3.5×10^9/L carried a median time to progression of 49.5 months, while cMBL with CLL lymphocytes >3.5×10^9/L presented a median time to progression of 63.5 months (P=0.020). CMBL were characterized by both lower beta2-microglobulin and soluble CD23 (sCD23) levels (P=0.04 and P=0.001) than Rai 0. Moreover cMBL showed both a significant higher prevalence of normal karyotype (50/98 [51%]] vs 38/116 [33%]) and a lower incidence of delq13 (27/98 [28%] vs 56/116 [48%] (P=0.0040). With regard to clinical outcome, 12/116 (16%) cMBL patients required treatment, while 42/135 (31%) Rai 0 cases underwent chemotherapy (P=0.008). Noteworthy, the time to treatment was significantly longer in cMBL patients (76 months vs 52 months, P=0.028). The slower disease kinetics was consistent with a longer TFS in cMBL compared to Rai 0 (74% vs 42% at 14 years, P=0.00002, Figure). Moreover, OS was significantly longer in cMBL (98% vs 71% at 18 years (P=0.04). Interestingly, some biological prognosticators such as IgVH status (P=0.01 and P=0.04) and ZAP-70 (P=0.0002 and P=0.007) as well as markers of tumor burden such as sCD23 (P=0.0006 and P=0.007) were confirmed to predict TFS both within Rai 0 and within cMBL. Finally, multivariate analysis, combining cMBL and Rai 0, documented that having a diagnosis of cMBL is the most important protective factor against the risk of treatment (P=0.00009), followed by a lower ZAP-70 (P=0.0001) and a normal karyotype (P=0.028). In conclusion, cMBL represents a biological and clinical distinct entity from Rai 0 with a more favorable outcome, displaying both a lower risk of treatment requirement and a longer OS.

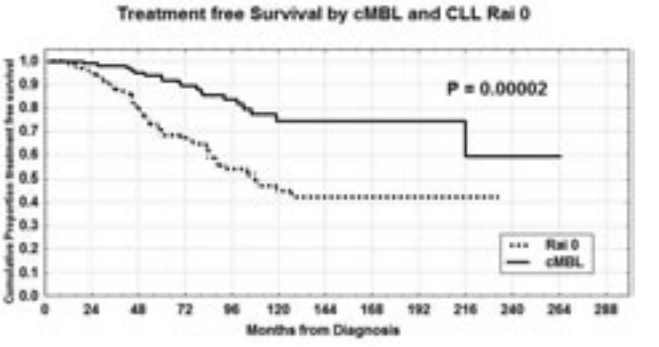

Figure 1.

PO-122

ANALYSIS OF IGHV REARRANGEMENT AND NOTCH1 MUTATIONS IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA ASSOCIATED WITH TRISOMY 12

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Introduction. Chronic Lymphocytic Leukemia (CLL) is a heterogeneous disease with variable prognosis and evolution. CLL can be classified into mutated or unmutated cases depending on the percentage of mutation in the immunoglobulin heavy chain (IGH) compared to germline sequence: unmutated CLL show a poorer survival and have high levels of expression of ZAP-70 and CD38 compared to mutated CLL. The majority of CLL cases have chromosomal aberrations, mostly represented by deletions of 11q23, 13q14, 17p13 or trisomy 12 (+12 CLL). Recently it has been observed that mutations in C-terminal PEST domain of NOTCH1 generate a truncated constantly active protein, always associated with +12 CLL and conferring a poor prognosis. *Methods.* CLL samples obtained from 165 patients at the time of diagnosis or within 2 years without treatment were subjected to flow cytometric analysis, cytogenetic investigation by FISH and MLPA analysis, and molecular assays. 21.8% of patients had +12 and represented our study population.

Total RNA was extracted from white blood cells and cDNA was tested for IGH mutational status using family-specific VH leader primers, in agreement with ERIC recommendations. PCR products were analysed by capillary electrophoresis and subsequently sequenced and compared to IGH germline sequence by IMGT/V-QUEST software. According to established procedures, sequences were compared to known stereotyped sequences available from several public databases, in order to detect stereotyped complementary-determining region 3 (CDR3). NOTCH1 mutations were investigated by direct sequencing of +12 CLL samples using primers for the PEST domain of the protein. *Results.* In our +12 CLL cohort, 71% of cases were unmutated while 29% were mutated; in the unmutated group 54.5% of cases carried stereotyped CDR3, while only one stereotyped CDR3 was found in the mutated group. As reported in literature, the expression of ZAP-70 and CD38 was significantly higher in unmutated +12 CLL compared to mutated +12 CLL. According to FISH results, we observed that 72.2% of cases had isolated trisomy 12 while remaining cases had more than a single chromosomal aberration. Concerning NOTCH1 mutations we observed two different mutations (P2515Rfs and V2537I) both confined to the PEST domain and restricted to unmutated cases. Four of 5 P2515Rfs mutations were observed in CLL with isolated +12, while the V2537I mutation was observed in a patient with complex charyotype. Interestingly, four of 5 P2515Rfs mutations were also observed in patients with stereotyped CDR3. *Conclusions.* In our +12 CLL patients NOTCH1 mutations were limited to unmutated cases, and preferentially found when +12 was the only chromosomal abnormality. The majority of cases presented a 2bp frameshift deletion originating a constantly active truncated protein while one patient presented the missense mutation V2537I, never previously reported in CLL.

PO-123

A NEW NOTCH1 MISSENSE MUTATION IDENTIFIED IN A B CHRONIC LYMPHOCYTIC LEUKEMIA (B-CLL) PATIENT HARBOURING TRISOMY 12 AND MYC ALTERATION

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Introduction. NOTCH1 is a member of a family of trasmembrane proteins acting as cell surface receptors and transcription regulators involved in cell differentiation, proliferation and apoptosis. Activating NOTCH1 mutations were firstly described in T-ALL patients and recently in about 10% of B-CLL at diagnosis, often associated with unmutated IgHV genes, trisomy 12, TP53 disruption, and progression to Richter's syndrome (RS). CLLs display a recurrent 2-bp frameshift deletion CT7544-7545 (P2515fs) affecting the proline, glutamine, serine and threonine (PEST) domain of NOTCH1. This mutation causes the degradation of the C-terminal domain, responsible for ubiquitinylation and degradation of NOTCH1 resulting in a more stable protein. In RS patients the frequency of P2515fs mutation is much higher (58%) than in CLL at diagnosis; other nonsense mutations all restricted to the PEST domain have been described. *Methods and results*. We report a 57-year old man with a Binet stage A B-CLL diagnosed in 1996. At disease progression on 2008, fluorescence in situ hybrydization analysis on peripheral blood showed a complex charyotype with at least 2 aberrant clones: nuc ish(8q24) x3 [90/300], nuc ish(12q13) x3 [90/300], nuc ish(13q14) x1 [90/300], nuc ish(17p13) x1 [210/300], ish (6q21,4,11q23) x2. The analysis of IgH mutational status revealed an unmutated rearrangement for V6-1*01 and J6*02 genes but the D gene was not detect. Direct sequencing of both DNA and RNA using primers for NOTCH1 PEST domain showed a new missense mutation 7609G>A causing the isoleucine substitution of valine (V2537I). Two years later rapid hyperleukocytosis, marked splenomegaly, hypercalcemia and LDH sieric level elevation were observed. Bone marrow aspirate analysis showed almost complete infiltration by vacuolated lymphoid cells with "blastoid" appearance, in accordance with the clinical hypothesis of RS trasformation. Cytofluorimetric analysis evidenced 2 populations, the first (82%) was CD10+/5-/38++/HLA-DR++,CD20-/23-/200-/11b-/103-/30-/lambda++ while the second (3%) was CD10-/5+partial/20+/HLA-DR+heterogeneous/lambda+. Molecular investigation confirmed the V2537I NOTCH1 mutation while no MYC rearrangement was observed by FISH. *Conclusions.* We describe a new missense mutation of NOTCH1 C-terminal PEST domain which has never been reported before in CLL but only in a case of early childhood T-ALL with MLL rearrangements and in patients with congenital left ventricular outflow tract malformations. As known MYC alterations and NOTCH1 mutations, prevalently in a mutually exclusive fashion, are associated with CLL transformation into RS. In this case, the previously detected MYC alteration disappeared at the occurrence of high grade transformation, suggesting that the persisting new V2537I NOTCH1 mutation may play a key role in RS evolution. Further investigations by CGH-array analysis are ongoing to better characterize this case.

PO-124

INCIDENCE OF CHRONIC LYMPHOCYTIC LEUKEMIA IN THE GENERAL POPULATION

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Introduction. Chronic Lymphocytic Leukemia (CLL) is the most frequent leukemia in the western countries (Rozman 1995) The incidence ranges between 2 - 6 cases/100000 pt-years (Linch, 1992; Brinker, 1982). These values are based on cancer registries data but these sources may be unreliable given the disease is often asymptomatic or diagnosis recorded only for in-hospital patients. In fact a higher incidence is reported in based-population survey (Zent, 2000). The reported median age at diagnosis between 65 to 72 years (Call, 1994, www.seer.cancer.gov) may be biased as well. For these reasons we tried to estimate CLL incidence and age at diagnosis in a population based investigation avoiding any selection bias. *Methods.* All individuals registered in Vicenza town census with a diagnosis of CLL between 1993 and 2010, were identified by searching the Veneto Region Health Service and the Hematology Department of Vicenza Hospital (the only referral centre for the metropolitan area) files. These files contain the diagnoses of all in- and outpatients referred to the Department, the CLL-related ICD-9 discharge codes (204.1 to 204.9) and the data of dead or moved people. We retained only CLL cases occurred in subjects belonging to a randomly chosen 15,055 healthy people cohort aged 18 to 65 years (median 43) enrolled from 1993 to 1996 in a clinical survey carried out in the Vicenza metropolitan area (Frezzato, 2010) and with a normal CBC count at enrolment. The diagnosis was based on standardized criteria (Cheson, 1988; Halleck *et al.* 2008) and confirmed by review of patient's clinical charts. All searches were authorized by the hospital IRB. *Results.* Nineteen CLL cases (12M/7F) were identified in the cohort. Total time of exposure was 219308 pt-years, median follow-up 14.56 years. Age-adjusted CLL incidence is estimated 6.86 cases/100000 pt-years (CI: 6.1 – 7.63) for the entire cohort, 23.2/100000 in older than 70 years. Mean age at diagnosis was 67.2 years (38.2 - 80.5). Seven subjects (37 %) were < 65 years, 4 (21%) older than 70. All the patients were asymptomatic at diagnosis, and 15 (79%) were in Rai stage 0. The mean follow-up was 54.6 months (4.2 - 136). Five patients needed therapy. One patient died for unrelated cause and 2 for disease progression 54 and 132 months after diagnosis. *Conclusions.* The estimated incidence of 6.86 cases/100000 pt-years is higher than reported so far. 37% of cases are younger than 65 years and this figure is slightly higher than 31% reported in 2004 to 2008 SEER analysis (www.seer.cancer.gov). Even if diagnosis may be done by chance in young people, the identification of these patients may be very important for a better knowledge of the disease progression and give some hints on the value of early diagnosis in order to therapeutic options. Three out of five patients who needed a treatment, were younger than 65 and had a Rai stage 0 disease at diagnosis. A longer follow-up could allow a more accurate evaluation of these issues.

PO-125

GENETIC ABNORMALITIES AND IGVH GENE REARRANGEMENT PRECEDING THE CLINICAL ONSET OF CHRONIC LYMPHOCYTIC LEUKEMIA

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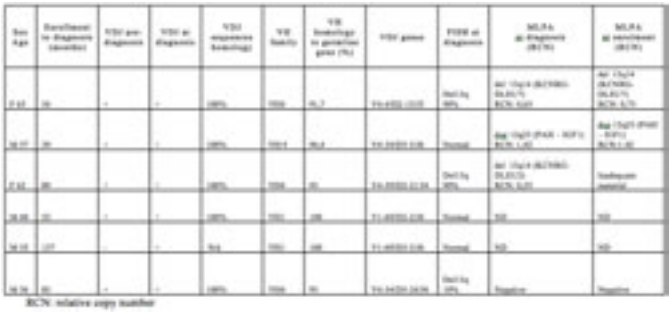
Introduction. The preclinical phase of CLL is largely unknown. A clonal IGVH rearrangement may precede by several years the appearance of CLL (Frezzato 2010, Landgren 2009) but genetic abnormalities, detected in more than 50% of cases at diagnosis (Dohner, 2000) have not been

studied in the preclinical phase. To this aim we searched for genetic abnormalities in people with a clonal B-cell population subsequently evolving in CLL. *Methods.* We identified 6 patients with a diagnosis of CLL, based on standardized criteria (Halleck, 2008), in a cohort of 15055 healthy (and with a normal CBC count) subjects, enrolled between 1993 to 1996 in an ongoing prospective clinical survey. A DNA sample stored at -30°C at the enrolment, was available for each of them. In 5 cases the same IGH gene rearrangement identifying the B-cell clone, was detected 39 to 89 months before CLL diagnosis (Frezzato, 2005; 2010). FISH analysis (Brothman, 2009) at diagnosis (ATM/SE 11, GLI/SE 12, DLEU P53/SE 17) was compared with Multiplex Ligation-dependent Probe Amplification (MLPA) analysis (Buijs, 2006; Al Zaabi, 2010) both on samples at diagnosis and on preserved DNA using MLPA P040 test kit, including set probes for 11q23 (ATM, 6 probes), chromosome 12 (9 probes), 13q14 (10 probes), 17p13.1 (p53, 7 probes) chromosomal regions, according to the manufacturer's protocol (MRC Holland). *Results.* A del(13q14) was identified at diagnosis in 3 and 2 patients respectively by FISH by MLPA. In one of them, MPLA revealed the deletion as well as 54 months before diagnosis. Moreover MLPA detected a chromosome 12 duplication, not detected by FISH, at diagnosis and 39 months before (see Table for details). *Conclusions.* The MLPA CLL-associated genetic alterations seem to be present, as well as IGH gene rearrangement, in an early phase of the disease, a long time before clinical diagnosis. They include the 13q14 DLEU1 locus deletion, proposed as a CLL-associated tumour suppressor gene (Ouillette, 2008) and, in 1 case, a dup 12q23 not identified by FISH, in 40% of the clonal population. Moreover they might evolve along time (pt 1, pt 6). Our results could prompt further investigations on the role of genetic abnormalities in the pathogenesis of CLL and in their clinical meaning.

Table 1.



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PO-126

CORTACTIN CONTRIBUTES TO B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA (B-CLL) AGGRESSIVENESS VIA THE RELEASE OF MMP-9 PROTEASE.

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Introduction. Cortactin is an actin-binding protein involved in several cell functions, i.e. the assembly and the organization of cytoskeleton. Its overexpression was observed in several human cancers and experimental data support the role of cortactin in metastatic capability through the regulation of cell motility and the release of matrix metalloproteinase-9 (MMP-9). The activity of this protein is regulated by its phosphorylation in Tyr residues by Src kinase family. We previously demonstrated that in leukemic cells from B-CLL patients the Src kinase Lyn is overexpressed, activated and involved in the resistance to apoptosis. Recently, we found that cortactin is overexpressed in patients with B-CLL. Here we investigated the involvement of cortactin in the release of MMP-9 and, therefore, in the progression of B-CLL. *Methods.* Blood samples were collected from 5 controls and 15 B-CLL patients. Informed consent was obtained according to the Declaration of Helsinki. Untouched peripheral blood B cells were purified using the RosetteSep for human B cells isolation kit. The samples that were used had at least 95% of normal CD19+ or neoplastic CD5+/CD19+ cells, as assessed by flow-cytometry. The purified B cells (2 106 cells/mL) were cultured in RPMI medium with or without CXCL12 (100 ng/mL) for the evaluation of MMP-9 production. MMP-9 release by neoplastic B cells was also investigated after cortactin silencing in 4 patients which expressed high level of cortactin. The protein was silenced by SMARTpool siRNA collection (Dharmacon, Thermo Scientific), according to the manufacturer's instructions. *Results.* By gelatin zymography we found that the release of MMP-9 by neoplastic B cells correlated to the expression of cortactin after 5 and 24-hrs culture. To investigate whether cortactin was involved in MMP-9 secretion in B-CLL, a cortactin-targeted siRNA silencing system was used to knockdown this protein in 4 patients with high cortactin expression. We found that following cortactin knockdown, leukemic cells showed a defect in MMP-9 secretion, as assessed by ELISA test. This protease also showed a decreased gelatinolytic activity in culture medium, confirming the hypothesis that cortactin is involved in the regulation of MMP-9 secretion in B-CLL malignant cells. Finally, we found that the incubation of leukemic cells with PP2, a Src kinase inhibitor, decreased Tyr phosphorylation level of cortactin and shut down the release of MMP-9 in culture medium, also following CXCL12 triggering. *Conclusions.* The overexpression of cortactin in neoplastic B cells and the correlation between cortactin levels, activity and MMP-9 release suggest a role of this protein in metastatic invasion and in the B-CLL aggressiveness. In addition, cortactin might represent a biomarker for diagnosis and prognosis and a target for new therapeutic strategies.

PO-127

BENDAMUSTINE IS A CYTOTOXIC AGENT USED IN HEMATOLOGY BUT ALSO RECENTLY IN SOLID TUMOR. A RARE CASE REPORT OF CONCOMITANT SQUAMOUS CANCER OF HEAD AND NECK AND CHRONIC LYMPHOCYTIC LEUKEMIA TREATED WITH BENDAMUSTINE AND RADIOTHERAPY

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Introduction. Bendamustine is an alkylating agent with hybrid activity that combines a purine-like benzimidazol and alkylating nitrogen mustard group and is actives in hematology neoplasms (LNH, LLC, myeloma, etc.) but also in solid tumors, like breast cancer and head and neck tumors. We describe a case of old patient that was treated with bendamustine for concomitant chronic lymphocytic leukemia (CLL) and a head/neck tumor. *Case report.* A 78 year old patient with CLL (stage B Binet / III RAI class.) was treated with chlorambucil for several years and recently he was in disease's progression. The patient same month ago noted a lesion in the left preauricular region that the scraping of the skin showed as a squamous cell carcinoma. He underwent surgery that confirmed this skin tumor. The deep margins were involved but he didn't practice any therapy. Progressively and rapidly the lesion relapsed arriving to determine an evident swelling involving the left parotid region and which was pushed towards the outer ear. At the same time the progression of the chronic leukemia therapy needs an effective and rapid therapy. In fact, the CT scan showed the lymph nodes enlarged in almost all the stations, and the level of hemoglobin and platelets gradually decreased. On the basis of recents works of literature about the use of bendamustine even in solid tumors and in combination with RT, the patient was treated with Rituximab and bendamustine associated with regional radiotherapy; with this combination of chemotherapy we are treating the squamous cancer in local evolution but also the chronic lymphocytic leukemia. The patient has started treatment and the first effects are encouraging. Bendamustine was administered as a 60-min 70 mg / m^2, intravenous infusion on days 1 and 2 of six 21-day cycles and with rituximab 375 mg/m^2, intravenous infusion for 3 hours. This therapy was well tolerated. *Discussion*. The cytotoxic agent bendamustine is indicated in several countries for the treatment of indolent non-Hodgkin's lymphoma and mantle cell lymphoma although it has clinical antitumor activity also in myeloma , breast cancer and head and neck tumors. While the precise mechanism of action of bendamustine is as yet unknown, it has limited cross resistance to other alkylating agents and appears to exert its antineoplastic effects via a different mechanism to that of other alkylating agents. The application of radiochemotherapy

with bendamustine is well tolerated also in patients with reduced of physical conditions. *Conclusion*. Combined treatment consisting of cytotoxic chemotherapy with Bendamustine and short term irradiation therapy is a successful palliative treatment of patients with progressive tumors of the head and neck besides LNH and LLC. This case report shows the efficacy of bendamustine in head and neck cancer in a patient with LLC and highlights a favorable toxicity profile.

PO-128

ALEMTUZUMAB MAINTENANCE MAY PROLONG CHEMOTHERAPY - FREE INTERVAL AFTER R-FC PROTOCOL IN CHRONIC LYMPHOCYTIC LEUKEMIA : OUR EXPERIENCE

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Introduction. Alemtuzumab (Alem) is a humanized monoclonal antibody against CD52 antigen, expressed in most human B and T lymphocytes. Alem is approved as therapy for patients suffering from B-CLL in which treatments with fludarabine phosphate, the second-line therapy for B-CLL, have failed or for patients with treatment-resistant (refractory) recurrent disease. The potential value of Alem in maintenance therapy of previously treated chronic lymphocytic leukemia (CLL) patients(pts) after they have achieved stable disease or partial remission with chemo or chemo-immunotherapy is discussed. *Methods.* we report our experience of 4 adult patients with B lineage CLL, Stage III Rye that was treated with R-FC for 6 cycles and subsequently, in complete remission, received Alem as manteinance. The following schedule of subcutaneous Alem was administered: a dose of 10 mg 3 times per week for 4 weeks, followed by 10 mg of Alem once every 2 weeks for a period of six month. With the exception of reversible grade 3 neutropenia, no unusual Alem - related toxicities, including opportunistic infections, were noted. The blood levels of CD19+ (B) cellswere extremely low without concordant suppression of CD4+(T) lymphocytes. Weekly surveillance for cytomegalovirus (CMV) by qualitative PCR-based assay was done and infection prophylaxis (trimethoprin-sulfamethoxazolo acyclovir/itraconazol) as well . Initial grade I-II local skin erythema and injection site pain disappeared in four weeks. Patients received standard prophylaxis with sulfamethoxizole and acyclovir with regular CMV monitoring by quantitative PCR. No symptomatic CMV reactivation were observed. No more infections occurred and the patient maintained a good partial hematological remission, with normal blood counts. These results suggest that low dose of Alem are indicated to develop a user-friendly Alem maintenance therapy for long-term control of patients with continuing CLL activity. The patient is now, after 2 years, still well (karnowsky 100%) in persistent complete remission, without opportunistic infections. *Discussion*. Alemtuzumab as a single agent is used in a novel maintenance schedule in previously treated chronic lymphocytic leukemia patients with the goal of delaying progression of disease. It is currently being investigated in other uses including subcutaneous administration for first line therapy and in a variety of combination and sequences, including fludarabina and other monoclonal antibodies, in both first and second line therapies. Adverse events usually include acute first dose reaction, hematological toxicity and infectious complications, particularly CMV reactivation. *Conclusion.* The manteinance with alemtuzumab treatment by low dose, subcutaneous administrations, may have a favourable toxicity profile and may provide prolonged disease control and chemotherapy-free intervals and survival in previously treated CLL patients in CR after R-FC.

PO-129

B-CELL RECEPTOR CONFIGURATION AND ADVERSE CYTOGENETICS ARE ASSOCIATED TO A HIGHER RISK OF AUTOIMMUNE HEMOLYTIC ANEMIA IN THE COURSE OF CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. Biological features related to the development of autoimmune hemolytic anemia (AHIA) in patients with chronic lymphocytic leukemia (CLL) may provide an important contribution to the understanding of the pathogenesis of autoimmune phenomena in this disease. *Design and Methods.* We retrospectively analyzed 585 CLL patients for the occurrence of AIHA. The clinical characteristics at CLL diagnosis and follow-up were available for all patients, cytogenetic analysis at the time of diagnosis was available in 409 patients, whereas the immunoglobulin heavy-chain variable (IGHV) gene status and B-cell receptor (BCR) configuration (HCDR3) were available in all cases. *Results.* AIHA occurred in 73 patients and it was significantly associated with an IGHV unmutated (UM) gene status (P<0.0001) and unfavorable [del(17)(p13) and del(11)(q23)] cytogenetic lesions (P<0.0001). Stereotyped HCDR3 sequences were identified in 173 out of 585 patients (29.6%) and were similarly represented among patients developing AHIA (28.7%) or not (29.6%). Of the stereotyped subsets, subset #3 was associated with a significantly higher risk of AHIA than the other HCDR3 configurations (P=0.004). When the analysis was restricted to UM patients, a strong association was found between AHIA and "truly" UM patients, defined as patients carrying a 100% identity with the germline configuration. Multivariate analysis showed that UM IGHV, del(17)(p13) and del(11)(q23), but not subset #3 - due to its marked association with UM IGHV - were the strongest independent variables associated with a risk of developing AHIA (P=0.03, P=0.004, and P=0.01, respectively). Based on this analysis, we generated a risk scoring system for AIHA development. Patients could be stratified significantly according to the presence of no (low risk), one (intermediated risk) or two (high risk) of the selected (unfavorable cytogenetics and UM IGHV status) risk factors (Figure 1). *Conclusions.* Our data indicate that CLL patients with an UM IGHV mutational status or carrying unfavorable cytogenetics can be characterized by a higher risk of developing secondary AHIA and suggest a possible role of specific stereotyped BCR subsets in some cases.

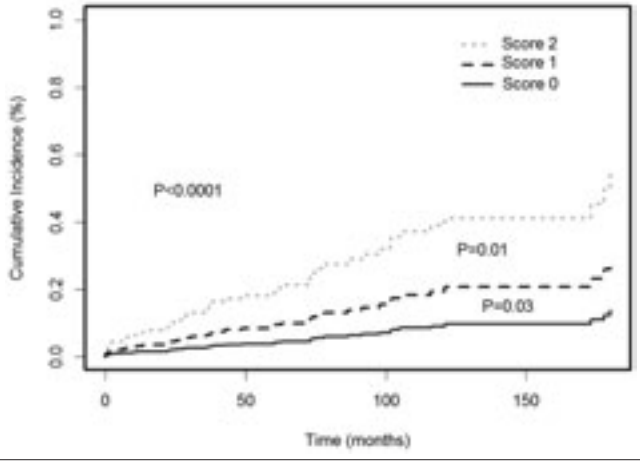

Figure 1.

PO-130

OCCASIONAL FINDING OF COMPLEX KARYOTYPE WITH INV(14)(Q11Q32) IS AN ALERT FOR HIDDEN T-CELL PROLYMPHOCYTIC LAUKEMIA

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Introduction. We describe a case of t-cell prolymphocytic leukemia (T-PLL), diagnosed after the occasional finding of inversion of chromosome 14 in course of screening for sterility. Case report. In February 2010, a 32 years old male performs screening for sterility. Cytogenetic analysis on peripheral blood shows a cellular clone with complex karyotype and paracentric inversion of chromosome 14 [inv(14)(q11q32)]. This cytogenetic alteration is related to mature T-cell lymphoproliferative disorders so cytogenetist sends patient to our attention. He has a good performance status and doesn't show any symptom. Clinical examination is negative. Complete blood count is in normal range with a lymphocyte count of about 5000/mm^2. Hepatic and renal function are good. Morphologic exam of peripheral blood shows prevalence of small lymphocyte. We decide to perform flow cytometric immunophetyping on peripheral blood showing a small pathologic T-cell population (57% of total lymphocyte) with this phenotype: CD3+, CD4-, CD8+, CD5+, CD2+, clonal restriction for TCRv 14. Same pathologic cellular population is found on bone marrow even if histological analysis doesn't show a clear lymphoid infiltration. Computed tomography (CT) doesn't show any alteration. Diagnosis of t-cell prolymphocytic leukemia is performed and we decide for "watch and wait" approach. During follow-up we assist to a progressive increase of peripheral lymphocyte and twenty-three months after diagnosis, lymphocyte count is 51000/mm^2; performance status is good. Furthermore clinical examination shows bilateral axillary and inguinal lymphoadenopathy (maximum diameter 2 cm). Lymph nodal and bone marrow biopsy confirm diagnosis. CT doesn't show other disease's localization. Discussion. T-PLL is a rare malignancy; it is most common type of mature t-cell leukemia but it accounts for only 2% of small lymphocytic leukemias in adults. A distinctive hematologic feature is a progressive rising of white blood cells. Most cases derive from T-cell CD4+; about 15% show a prevalence of CD4-/CD8+ cells and a third of cases shows a prevalence of CD4/CD8 coexpression. The most distinctive cytogenetic abnormality is inv(14)(q11q32). T-PLL has an aggressive course and limited therapeutic options with median survival <1 year (Matutes *et al*, 1991). Anti-CD52 monoclonal antibody alemtuzumab alone resulted in overall response rates between 51 and 76 % but median survival is still short (Dearden *et al*, 2001; Keating *et al*, 2002). Stem cells transplantation after alemtuzumab may provide better results over alemtuzumab alone with long term survival in some patients (Krishnan *et al*, 2010). *Conclusions.* With this case we express importance of research lymphoprolipherative disorders in presence of particular cytogenetic alterations, found in course of kariotype analysis, performed for other reasons (i.e. sterility tests).

PO-131

IL6 AND RANTES /CCL5: TWO PRO-INFLAMMATORY CYTOKINES INVOLVED IN LGL LEUKEMIA

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Introduction. Large granular lymphocyte (LGL) leukemia is a rare lymphoproliferative disorder characterized by clonal expansion of large lymphocytes with cytotoxic activity. Although the etiology of the disease is largely unknown, LGL leukemia is often referred to an inflammatory condition and many reports strongly support the role of an antigenic stimulation by a foreign infectious agent as the initial step. In this contest, we have recently demonstrated that IL6, a major activator of JAK/STAT pathway, is overexpressed in LGL leukemia patients, suggesting a role of this cytokine in the activation of STAT3 and the consecutive high expression of its target genes.

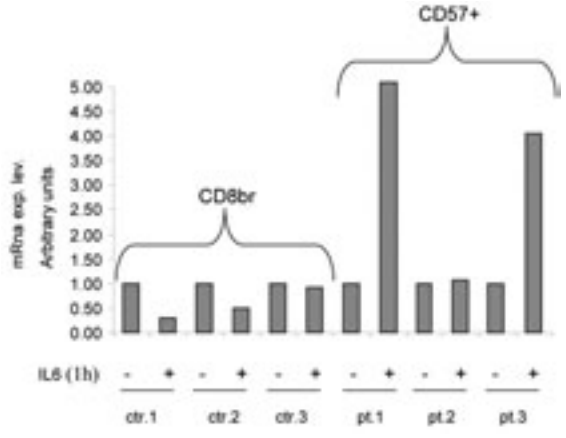

Figure 1.

Among these, CCL5 is an important mediator of acute and chronic inflammation with a well defined chemoattractant property, which is reported to be overexpressed in patients' plasma. Since IL6/STAT3 axis is constitutively activated in LGL leukemia patients and CCL5 is overexpressed, we investigated whether IL6/STAT3 axis modulates CCL5 expression. In addition, we investigated the role of bone marrow mesenchymal stromal cells (MSCs) in the compartmentalization and survival of leukemic LGLs through IL6 release. *Methods.* LGLs from 20 patients and 5 healthy donors were highly purified from PBMC. Levels of CCL5 and IL6 were analyzed by Real Time-PCR assay in purified cell subsets (LGL and no-LGL PBMC) at resting condition and after stimulation. By ELISA test we also evaluated CCL5 and IL6 in patients' plasma and in cell culture supernatants. Moreover, we set long term cultures of MSCs obtained from bone marrow blood of patients with LGL leukemia and normal controls and we analyzed normal and neoplastic cell migration and survival in the presence of conditioned medium derived from primary cultures of MSCs. *Results.* First of all we confirmed by ELISA test that CCL5 was overexpressed in plasma of patients with respect to controls (patients: 4000±501 pg/mL SE vs controls: 1429±52 pg/mL SE $P<0.05$). Analyzing the CCL5 mRNA levels in neoplastic LGLs with respect to controls, we found CCL5 overexpression is specific of neoplastic cells. Moreover, we found that IL6 is able to stimulate the expression of CCL5 (Figure 1), supporting the role of IL6 in regulation of CCL5 level. When we investigated the levels of IL6, we observed that's increased in no-LGL PBMC suggesting a role for microenvironment cells in the release of IL6 and in the biological activity of neoplastic cell. Finally we found that MSC released in IL6 culture medium and were able to stimulate the survival and migration of LGLs. *Conclusions.* Our results support the hypothesis that the overexpression of IL6 and the alteration of JAK/STAT pathway contribute to the overexpression of CCL5 which could play a role in the pathogenetic processes taking place in LGL leukemia. We also suggest a promotive role of MSCs in the mechanisms leading to LGL progression trough the generation of chemotactic stimuli, the secretion of IL6 and triggering of CCL5.

PO-132

USEFULNESS OF UNICEL DXH 800 CELL POPULATION DATA IN THE DESCRIPTION OF MORFOLOGICAL FEATURES OF LYMPHOCYTES IN CLL AND NHL

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Introduction. Lymphoproliferative disorders (LD) are characterized and described by lymphocyte population with heterogeneous morphological features both in optical microscopy revision and in flow cytometry. Our laboratory is daily committed to discover, treat and follow-up oncohematological patients. In this communication we present data regarding some CLL and NHL patients. Several literature report the clinical usefulness of Cell Population Data (CPD) provided by Beckman Coulter hematology analyzers. Abnormal values of CPD correlate with morphological abnormalities of leukocytes. *Methods.* 27 CLL and 6 NHL untreated patients blood samples were collected in K3EDTA tubes and analysed on Coulter UniCel DxH800 that performs leukocytes differential with the Flow Cytometric Digital Morphology (FCDM) technology, based on the measurements of Volume (V), Conductivity (C) and 5-angle Scatter light laser (MALS, UMALS, LMALS, LALS, AL2) on cells in their native state. Mean and standard deviation of FCDM measurements are collected in 56 CPD. Normal CPD values were computed from a 42 normal samples. Statistical comparative test were performed with MedCalc software. *Results.* Following what is reported in the literature, we confirmed that Mean Lymphocyte (LY) volume (MV-Ly) is significantly (P<0.001) lower in CLL (80.6±8.2) than in normals (85.7±3.8); Volume standard deviation (Vsd_LY) is 16.6±3.2 in CLL and 13.4±0.9 in normals (P<0,0001). We looked also at nucleus/cytoplasm heterogeneity of LY and we found significant differences in the sd of the Conductivity (Csd-LY) that was 12.1±3.7 in CLL and 8.2±1.5 in normals. We performed ANOVA analysis in order to find any difference in CPD that was able to explain the different morphological features of LY in CLL and NHL. Mean value of UMALS (upper-medium-angle light scatter) was statistically different (P<0.001) in CLL (34.67), NHL (42.8) and normals (60.4); mean value of LALS (low-angle light scatter) was statistically different (P<0.001) in CLL (52.8), NHL (45.3) and normals (63.4). *Conclusion.* Laboratory hematologist are currently driven to optical microscopy by abnormal numerical values and/or suspect flags that, even if are generally triggered by abnormal cells, are unable to give information about different cellular morphologies. Literature reports show that CPD computed by UniCel DxH800 are able to describe abnormal leukocytes features that give useful information to microscope revision. In this brief report we presented the preliminary data of our study on CLL and NHL untreated patients. MV-LY, Vsd-LY and Csd-LY are significantly different in normal and CLL sample showing the difference for the size of the cells and the nucleus/cytoplasm ratio. The specific cytoplasmic features of the LY in NHL, CLL and normal samples were clearly reported as significant differences in the values of UMALS and LALS. Further studies will be done in order to define cut-offs of the CPD values that could be used in triggering specific comments useful for the daily hematological routine.

PO-133

PRO-APOPTOTIC EFFECT OF A NEW SMAC-MIMETIC ON B-CLL CELLS *IN VITRO*

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Introduction. Pharmaceutical research is extensively focusing on the apoptotic process and dysfunctions in its regulation in many human hematological diseases like B-CLL. The family of Inhibitor of Apoptosis Proteins (IAPs) was considered putative target for pro-apoptotic drugs in oncology. Surely, the most caspase-connected human IAP is the X-Inhibitor of Apoptosis Protein (XIAP). XIAP is capable of binding caspase 9 (the initiator caspase) and both caspases 3 and 7 (the executioner caspases). Its activity is endogenously antagonized by second mitochondria-derived activator of caspases (Smac), and also by small molecules mimicking Smac that can induce apoptosis in tumor cells activating the extrinsic (or death receptor-dependent) path as well as the intrinsic (or mitochondrial) path. XIAP is reported to be overexpressed in B-CLL. Here we describe the activity of a new Smac mimetic monomeric compound (Smac66) on B-CLL samples and healthy donors alone or in combination with proteasome inhibitor and Tumor necrosis factor–Related Apoptosis–Inducing Ligand (TRAIL). *Methods.* The pro-apoptotic effect of Smac66 10 uM was evaluated after 16 hours of treatment in lymphocytes isolated from 33 B-CLL patients by flow cytometry with annexin V/PI staining. Gene expression profiling (GEP) data were generated on 6 CLL patients in 3 replicates for both treated and untreated conditions by means of Gene 1.0 ST array (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's protocol. Data were acquired using the GeneChipÒ Operating Software (GCOS v1.4, Affymetrix). Robust Multi-Array average (RMA) normalized and log2-transformed values were obtained by means of Expression Console Software (EC v1.1, Affymetrix). Supervised analysis (Gene@Work) was carried out comparing Smac66 treated versus untreated CLL samples.Functional annotation analysis was performed by DAVID 6.7 tool. *Results.* Smac66 induced an average apoptosis in B-CLL of 42±19.7% SD, versus 9.9±5.8% SD in healthy donors demonstrating a significantly higher cytotoxic effect in B-CLL samples than in controls. No significant synergistic effect was observed in combined treatment with proteasome inhibitor nor with TRAIL. GEP supervised analysis evidenced 44 upregulated and 89 downregulated genes in Smac66 treated cells. A significant fraction of the modulated genes was involved in the regulation of apoptosis and cell death. Regulation of transcription, protein kinase activity, myeloid cell differentiation, cell-cell adhesion and lymphocyte activation were also deregulated functions by Smac66 treatment. *Conclusions.* The new compound Smac66, used as single agent *in vitro*, is highly active against B-CLL cells, showing modest toxicity on normal peripheral blood cells, thus suggesting a promising therapeutic potential as a new class of antileukaemic drugs. GEP results provide new insights into the molecular pathways deregulated by Smac66 to be further investigated.

PO-134

B CELLS FROM CHRONIC LYMPHOCYTIC LEUKEMIA UNDERGO APOPTOSIS FOLLOWING MICROTUBULE DEPOLYMERIZATION BY NOCODAZOLE

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Introduction. B-cell Chronic Lymphocytic Leukemia (B-CLL) is characterized by the accumulation of clonal CD19+/CD5+ B lymphocytes blocked in G0/G1 cell-cycle phase and by abnormal distribution of proteins which regulate cytoskeletal functions. Since microtubules are cytoskeletal subunits, it is worth of note that neoplastic B cells are significantly sensitive to microtubules inhibitors. Nocodazole is a synthetic anti-neoplastic agent that favours microtubule depolymerization and induces significant structural changes in tubulin, thus impairing microtubule dynamics and blocking cell-cycle progression. Data from the literature suggest that nocodazole induce apoptosis of B-CLL cells through regulation of Bcl-2 expression and phosphorylation. In this project, we investigated the effect of nocodazole in leukemic cells from B-CLL patients. *Methods*. In this study we investigated 70 B-CLL patients, 10 healthy controls, several cell lines (Jurkat, K562, and Raji) and mesenchymal stromal cells (MSCs) obtained from healthy donors and B-CLL patients. The cells were incubated with and without nocodazole (16uM) to assess cell viability using Annexin V/PI assay and to analyze proteic expression pattern by western blotting analysis. *Results.* In this study we observed that only neoplastic B cells, but not other cell types (Jurkat, K562, and Raji), underwent apoptosis following 24, 48 and 72h nocodazole exposure (cell viability of normal vs leukemic cells at 24h: 98±6% vs 57±10%, P<0.0001; Student's t test). Moreover, in B-CLL samples with a discrete number of T cells, we found that nocodazole killed B cells but it did not affect T cells (cell viability after 24h nocodazole treatment: 30±23% vs 82±12%). Data were also confirmed on B and T cell subsets by western blotting analysis of cleaved PARP. Since B-CLL cells are protected from apoptosis when treated with CD40L, plasma and/or co-cultured with MSCs, we wanted to investigate whether nocodazole was able to overcome these protective effects. We observed that B cells rapidly died, regardless of the presence of MSCs plus CD40L or plasma. Finally, western blotting analysis showed that the 24h nocodazole treatment decreased phosphorylation at pTyr396 Lyn active site (P=0,008, Student's t test) in B-CLL cells, thus blocking the kinase activity, but not Lyn proteic expression. *Conclusions.* In this work we observed that nocodazole induces a selective apoptosis of B-CLL leukemic cells, without affecting T and MSC cells of B-CLL patients, normal B cells from healthy donors as well as different cell lines (Jurkat, K562, and Raji). This effect seems to be a consequence of a down-regulation of Lyn kinase activity, through dephosphorylation and inhibition of its active site pTyr396. Our *in vitro* results suggest a new role for nocodazole as therapeutic compound for B-cell Chronic Lymphocytic Leukemia therapy.

Myelodysplastic Syndromes

PO-135

NUP98/NSD1 FUSION IN A CASE OF MDS PRESENTING AS HEMOLYTIC ANEMIA

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Introduction. The "promiscuous" NUP98 gene is recurrently involved in chromosome translocations with at least 28 partners in primary and therapy-related MDS and AML, in T-ALL, and in blastic phase chronic myeloid leukemia (BP-CML). One of the most frequent NUP98 translocations is cryptic t(5;11)(q35;p15), producing NUP98-NSD1 fusion. *Methods. Case report.* In April 2011 a 36 year-old male was referred because of fatigue, tachycardia and jaundice. In 2007 testicular seminoma had been treated with orchiectomy and local radiotherapy. Upon referral, hemolysis was documented by: indirect bilirubin 6.5 mg/dl, haptoglobin 6 mg/dL, reticulocytes 74.000/mm^2, LDH 1346 UI/L. Direct and indirect Coombs tests were negative. Seric pyruvate kinase was low (20.2 mU/mL). RAEB-2 was diagnosed on bone marrow biopsy and morphology. Conventional cytogenetics, FISH screening for recurrent chromosome changes in MDS/AML and mutational analysis were performed. The Affymetrics technology (Cytogenetics Whole Genome 2.7M assays) was used to analyse CNV and UPD on DNA extracted from bone marrow and saliva. As the patient rapidly evolved to frank AML, he underwent treatment according to the 3+7 protocol, received a haploidentical transplant but died of pneumonia on day +64. *Results.* At diagnosis: Karyotype was normal: 46,XY. FISH showed a NUP98 rearrangement in 90% of nuclei. Double-colour double-fusion FISH combining RP11-348A20 and RP11-3234F16 clones for NUP98 and CTC-549-A4 for NSD1 detected cryptic t(5;11)(q35;p15)/NUP98-NSD1. Molecular analysis revealed in-frame fusion between nucleotide 1443 (exon 11) of Nup98 (NM_139131.1) and nucleotide 3935 (exon 7) of NSD1 (NM_022455.4). Mutational analysis for NPM1 exon 12 and FLT3-ITD and 835 mutations was negative. SNPs did not detect any acquired genomic variations in bone marrow. After induction: bone marrow showed partial response. Interphase FISH detected 9% of nuclei with NUP98 translocation. RT-PCR confirmed NUP98-NSD1 fusion was present. Pyruvate kinase was normal suggesting the deficiency at diagnosis had been acquired and was associated with the leukemic clone. *Conclusion.* In 2004 we described the first case of NUP98-NSD1 positive RAEB. Here we report another case of which was probably induced by radiotherapy. Clinical, hematological, and morphological features in both cases were presence of immature ortochromatic erythroblasts in peripheral blood, and marked erythroid dysplasia in bone marrow, suggesting this NUP98 rearrangement predominantly targeted the erythroid line. In the present case an acquired pyruvate kinase deficiency was responsible for the unusual presentation as frank hemolytic anemia. No microdeletions at SNPs analysis suggested the PK deficit was not related to gene haploinsufficiency but rather to a functional effect. Surprisingly no additional genomic variations were detected by SNPs in this treatment induced MDS/AML. *Acknowledgements.* FCRP: 20.12.0108.021

PO-136

AZACITIDINE (AZA) FOR RELAPSED HIGH RISK MYELODYSPLASIC SYNDROME AFTER INTRABONE CORD BLOOD TRANSPLANTATION: A CASE REPORT

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Introduction. Relapse is a major cause of treatment failure after allogeneic hematopoietic stem cell transplantation (HSCT) for acute myelogenous leukemia (AML) and myelodysplastic syndrome (MDS), and usually prognosis is very poor. The hypomethylating agent Azacitidine (AZA) could exert an antileukemic effect on growing leukemic clone and restore a donor chimerism. *Methods.* We report the case of a 67 years old man with secondary AML, who was initially treated with intensive chemotherapy obtaining a complete remissione (CR). For a first relapse he was treated with AZA and subsequently with a reinduction therapy.

The patient was transplanted in second CR on 9th December 2009 with an HLA antigen 5/6 matched cord blood (CB) unit delivered by intrabone injection. The mismatch was on locus B antigen and the total nucleated cell count before thawing was 1,65 x 10^7/Kg. The patient was conditioned with Thiotepa, Fludarabine, Ciclofosfamide and ATG. Graft Versus Host Disease (GVHD) prophylaxis consisted in cyclosporine 1 mg/Kg from day -7 and mycophenolate mofetil 15 mg/Kg from day + 1 to day + 28. Neutrophil (> 500/mm^2) and platelet (> 20x10^3/mm^2) engraftment was observed on day + 33 with a donor chimerism of 100%. Since at the 3rd month the donor chimerism was reduced to 87,7%, we started the tapering of cyclosporine that was definitively withdrawn at 6th month. On the 23th month after HSCT, donor chimerism was 67% and it was associated with worsening pancytopenia. Bone marrow analysis showed a picture of MDS/RAEB-II (IPSS-INT 2) recurrence therefore AZA at standard dose, 75 mg/mq/daily for 7 days every month, was started. *Results.* The response to AZA was first assessed after completing 4 AZA courses. The patient obtained a morphologic CR (no cytogenetic or molecular markers were present) and the donor chimerism was 100%. The monitoring of donor chimerism on bone marrow and the increasing of haemoglobin, neutrophils and platelets values during AZA treatment are shown in Figure 1. The drug was very well tolerated with no extramedullary toxicities and with mild neutropenia without increase in the rate of infectious complications. No GVHD signs/symptoms were developed. The patient is still on therapy and in complete hematologic remission after 5 cycles of AZA. *Conclusions.* Our observations suggest that Azacitidine may represent a promising drug to treat minimal residual disease (MRD) or MDS/AML recurrence after HSCT and to restore donor chimerism.

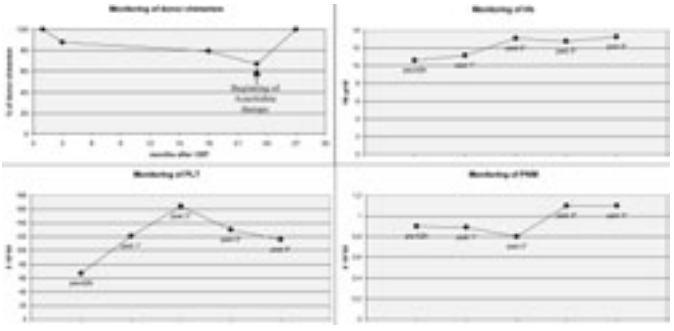

Figure 1.

PO-137

AZACITIDINE IN MYELODYSPLASTIC SYNDROMES: RETROSPECTIVE EVALUATION OF LONG-RESPONDER PATIENTS

Clissa C,[1] Finelli C,[1] Follo MY,[2] Curti A,[1] Paolini S,[1] Papayannidis C,[1] Mongiorgi S,[2] Parisi S,[1] Abbenante MC,[1] Bosi C,[3] Manzoli L,[2] Martinelli G,[1] Cocco L,[2] Baccarani M[1]

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Introduction. Azacitidine (AZA) has proven effective in myelodysplastic syndromes (MDS). The duration of haematological response is limited (median 13.6 months) (Fenaux, 2009), although some patients (pts) show a prolonged response. These data prompted us to retrospectively analyse our MDS pts treated with AZA, in order to enucleate long-responder pts (duration of response ≥20 months). METHODS. From September 2004, in our Institution, 52 MDS pts (40 males), median age: 70 (37-85) yrs, were treated with AZA, following 4 different treatment regimens: 9 pts received the AZA 7 regimen (AZA 7: 75 mg/sqm/die SC for 7 days/28 days); 6 pts received the combination of AZA 7 with valproic acid and all-trans-retinoic acid ; while 25 and 12 pts respectively received the alternative regimens AZA 5-2-5 and AZA 5 (Lyons, JCO 2009). 37 pts (71%) showed a IPSS high-risk MDS, while 15 pts (29%) with IPSS low-risk MDS received AZA because of refractoriness or ineligibility to erythropoietin, or secondary MDS. Moreover, as our group (Follo, 2009) demonstrated that phosphoinositide-phospholipase C (PI-PLC) beta1 may represent a target for AZA, we quantified the degree of PI-PLCbeta1 methylation and gene expression before and during AZA administration. RESULTS. 9 pts (17.3%) showed a prolonged hematologic response (≥ 20 months). Pre-treatment clinical and haematologic features of long-responders: sex (M/F): 4/5; median age: 69 (52-84); WHO: RCMD-RS: 1 pt; RAEB-1: 1 pt; RAEB-2: 7 pts; IPSS risk: low: 1 pt; int-1: 2 pts; int-2: 5 pts; high: 1 pt; IPSS cytogenetic risk: low: 7 pts; interm: 1 pt; high: 1 pt; ECOG: 0-1: 8 pts, ≥ 2: 1 pt; transfusion need (N° U)/8 weeks: < 4 : 4 pts; ≥ 4 : 5 pts; time from diagnosis (months): < 6 : 6 pts; ≥ 6 : 3 pts. Therapeutic regimen: AZA 7: 3 pts; AZA 5: 3 pts; AZA 5-2-5: 3 pts. Therapeutic response: median number of cycles: 19 (8-59); median time to 1st response: 3 (2-6) months; type of response: Complete Remission (CR): 3 pts; Hematologic Improvement (HI): 6 pts; cytogenetic remission: 1 pt; median duration of response: 30 (24-66) months; doubling of platelet count after 1st cycle: 4 pts; toxicity (grade > 2): 3 pts; 4 pts are still maintaining hematologic response, 3 pts are still alive but discontinued treatment because of disease progression, and 2 pts died (1 for AML and 1 for cachexy). Median survival (from the start of AZA): 38 (25-103) months. All the pts showed an increase in PI-PLCbeta1 expression, that was maintained along with the hematologic response. 15 pts (28.8%) showed a short-lived reponse (< 20 months), 5 pts (9.6%) show a shorter response but are still on treatment, 2 pts underwent allogeneic transplantation after 5 and 10 months. 7 pts (13.4%) are not evaluable for response (< 6 cycles), and 14 pts (26.9%) did not respond to AZA. *Conclusions.* Our data show that a limited but significant fraction of MDS pts show a long-lasting hematologic and molecular response to AZA.

PO-138

5-AZACYTIDINE FOR THE TREATMENT OF CHRONIC MYELOMONOCYTIC LEUKEMIA: RESULTS OF AN ITALIAN RETROSPECTIVE STUDY

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Introduction. Chronic myelomonocitic leukemia (CMML) is a rare myeloproliferative-myelodysplastic syndrome, characterized by poor response rates to conventional treatment. Two hypomethylating agents, 5-azacytidine (5-AZA, VidazaTM, Celgene Corp.) and decitabine, have been approved by FDA for CMML. *Methods.* We retrospectively collected clinical data of 31 patients (23 males/8 females, median age 69 years, range 53-84) diagnosed with CMML (13 type-1 and 18 type-2) and consecutively treated with 5-AZA at 8 Italian Hematology Centers between 2005 and 2011. 5-AZA was administered at 75 mg/m^2 or 50 mg/m^2 daily for 7 days (21 patients and 1 patient), or at 100 mg daily for 5 or 7 days (2 and 7 patients, respectively), every 4 weeks. Response was assessed after a median of 4 cycles (range 2-7), according to the modified International Working Group 2006 criteria. *Results.* At the time of therapy start, mean Hb level was 9.9 + 0.3 g/dL (SEM), white blood cell counts: 12.9 + 1.8 x10^9/L , monocytes: 3.9 + 0.8x10^9/L, neutrophils: 5.2 + 0.9 x10^9/L, platelets: 85 + 13x10^9/L. Karyotype was normal in 23 patients (74%), 4 patients had a trisomy 8, 1 complex karyotype, 1 -Y, 1 ins(19;2). IPSS was low in 3 pts, Intermediate-1 in 9, Intermediate-2 in 14, and high in 3 patients. Eleven patients received cytoreductive treatment with hydroxyurea prior to 5-AZA start. Patients started 5-AZA at a median of 6 months from initial diagnosis (range 0-43.6 months), and were treated for a median of 6 cycles (range 2-31). Treatment was generally well tolerated (only 1 patient experienced grade 4 anemia, 1 grade 4 thrombocitopenia and 1 grade 4 anemia and thrombocytopenia). Overall response rate was 51%, with a high remission rate (45%, 13 complete remission and 1 partial remission), and 6% haematological improvement (HI, 2 pts). The disease was stable (SD) in 7 patients (23%) while 8 patients resulted resistant (26%). Response to 5-AZA was associated to monocyte counts below 2x10^9/L at diagnosis and at treatment start (P=0.01 and 0.05, respectively). Estimated median OS was 37 months from treatment start. Five patients (16%) progressed to acute leukemia at a median of 12.7 months (range 7.4-20.2) from 5-AZA start. Patients responding to 5-AZA (including CR, PR and HI) had a significantly better survival than those with SD or progression (37 vs 16 months, P=0.07). There were no survival differences grouping patients according to kary-

otype, platelets counts, IPSS, WPSS, and CMML type 1 versus 2, and 5-AZA dose. Improved survival was associated with monocyte counts less than $10x10^9$/L and peripheral blood blasts below 5% at treatment start (P=0.04 and 0.01 respectively). There was a trend for worse survival for transfusion-dependent patients and for those treated with hydroxyurea prior to 5-AZA (P=0.06 and 0.07 respectively). *Conclusion*. 5-AZA treatment in CMML patients is safe and effective, with better response and survival associated with limited disease burden.

PO-139

ERYTHROPOIESIS-STIMULATING AGENTS (ESAS) IN MYELODYSPLASTIC SYNDROMES: RETROSPECTIVE EVALUATION OF LONG-RESPONDER PATIENTS

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Introduction. Erytropoiesis-stimulating agents (ESAs) are effective in 20-30% of patients (pts) with Myelodysplastic Syndromes (MDS), but response rates are higher when pts are selected on the basis of clinical and hematologic parameters: baseline serum erythropoietin (EPO) level <200-500 IU/L, , <10% marrow blasts, IPSS risk low or INT-1, shorter interval between diagnosis and treatment. The median duration of the response to ESAs is approximately 2 yrs, and a significantly longer response duration is associated with: major response (following IWG criteria), < 5% marrow blasts, absence of multilineage dysplasia (Jadersten, 2005; Park, 2008). These data prompted us to retrospectively analyse our MDS pts treated with ESAs, in order to enucleate long-responder pts (duration of response ≥20 months). METHODS. From October 2001, in our Institution, 69 MDS pts (50 males), median age: 78 (42-92) yrs, with Hb level <10 g/dL, IPSS risk low or INT-1 , and baseline serum EPO < 500 mIU/mL, were treated with epoetin alpha (EPO), at a starting dose of 40-80.000 IU s.c. per week (once or twice weekly), for a minimum of 12 weeks. Hematologic response was defined according to revised IWG criteria (Cheson, 2006). In a subgroup of pts we also studied the expression of several genes involved in inositide signaling. RESULTS. 34 pts (49.3%) showed a prolonged hematologic response (≥20 months). Pretreatment clinical and haematologic features of long-responders: sex (M/F): 21/13; median age: 77 (42-90) yrs; WHO diagnosis: RA: 22 pts; RARS: 6 pts; RCMD: 1 pt; RAEB-1: 5 pts.; IPSS risk: low . 25 pts, INT-1: 9 pts; WPSS risk: very low: 16 pts (47.1%), low: 12 pts (35.3%); INT: 3 pts (8.8%); high: 3 pts (8.8%); IPSS cytogenetic risk: low: 33 pts; INT.: 1 pt ; pre-treatment transfusions: 11 pts (32.4%); high pre-treatment transfusion need (>4 units/8 weeks): 6 pts (17.6%); long interval from diagnosis to the start of EPO (> 6 months) : 20 pts (58.8%). Starting weekly EPO dose: 80.000 U: 20 pts; 40.000 U: 14 pts. Outcome: median time to response: 8 (4-32) weeks; type of response: Complete Response (CR): 14 pts (44.1%); Haematologic Improvement (HI): 19 pts (55.1%); median duration of response: 47.5 (20-125) months; 15 pts (44.1%) were able to shift to a lower maintenance dose. Relapse occurred in 7 pts (20.6%) (in 2 pts because of disease progression) after a median of 48 months. 24 pts are still alive, 5 pts died (none of them for AML), and 5 pts were lost at follow-up. Median survival (from the start of EPO): 51 (22-128) months. 13 pts show a shorter response but are still on treatment , 12 pts showed a short-lived reponse (< 20 months), and 10 pts did not respond to EPO. *Conclusions*. Our data show that, although in MDS the duration of response to EPO is limited, a substantial fraction of pts may show a long-lasting response, and that, unexpectedly, some of them may show unfavourable pre-treatment prognostic features (WPSS risk, high transfusion need).

PO-140

T-CELL RECEPTOR REPERTOIRE KINETIC DURING AZACITIDINE TREATMENT

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Introduction. We have previously shown that patients with myelodysplastic syndromes (MDS) show an increased frequency of T cell expansions within their T-cell receptor (TCR) repertoire, which are mostly polyclonal in the CD4+ subset and oligoclonal in the CD8+ one. Azacitidine represents one of the best options for MDS and, beside the well known effects on bone marrow precursors, has been demonstrated to potentially influence T cell polarization. The aim of this study is to monitor the kinetic of the TCR repertoire during Azacitidine treatment in order to explore its potential ability not only to restore the hematopoietic function but also to reverse the immune derangement typical of these patients. *Methods*. Our study is based on a flow cytometric analysis performed on the peripheral blood of 8 patients (4 with MDS and 4 with AML with multilineage dysplasia) and 30 normal controls. Each patient is evaluated at baseline and then every 3 cycles of Azacitidine. TCR repertoire analysis is based on a panel of 24 beta variable (BV) family-specific antibodies. A BV expansion is defined as any value of BV family expression higher than the mean + 3 standard deviations calculated in controls. *Results.* Our patients had a median of 3 assessments during their treatment with Azacitidine. At baseline, in CD4+ cells 5 patients did not show any lymphocyte expansion while 3 of them showed a single BV expansion. One of these expansions disappeared after 3 cycles while 2 were stable during treatment. When reassessed 2 of the patients showed each the appearance of 3 new BV expansions, which however disappeared at the following evaluations. Overall CD4+ expansions during the all period of study were 9 (3 at baseline and 6 emerged during treatment) and their size ranged from 7.1% in BV 13.6 to 23.9% in BV 11. Within the CD8+ subset, at baseline 5 out of 8 patients showed at least one T cell expansion. In details 2 patients showed a single expansion while 3 of them displayed 2 different BV expansions. Of these 8 baseline expansions 5 were stable during treatment, while two of them quickly disappeared. Noteworthy, one of these expansions which had disappeared in a patient in remission reappeared at disease relapse. Three patients showed the appearance of a single BV expansion during treatment, which once again was usually transient. Overall CD8+ expansions during the all period of evaluation were 11 (8 at baseline and 3 emerged later) and their size ranged from 2.5% in BV 5.3 to 40.4% in BV 11. *Conclusions:* Our preliminary data confirm in MDS patients an increased frequency of T cell expansions, which appear to be tendentially stable during Azacitidine treatment in both CD4+ and CD8+ cells, whereas expansions emerging during therapy are usually transient. Noteworthy a CD8+ expansion which had disappeared after remission, re-emerged at relapse, thus suggesting a possible link with the disease evolution. A molecular evaluation based on CDR3 spectratyping will further dissect the impact of Azacitidine on the degree of immune derangement typical of MDS.

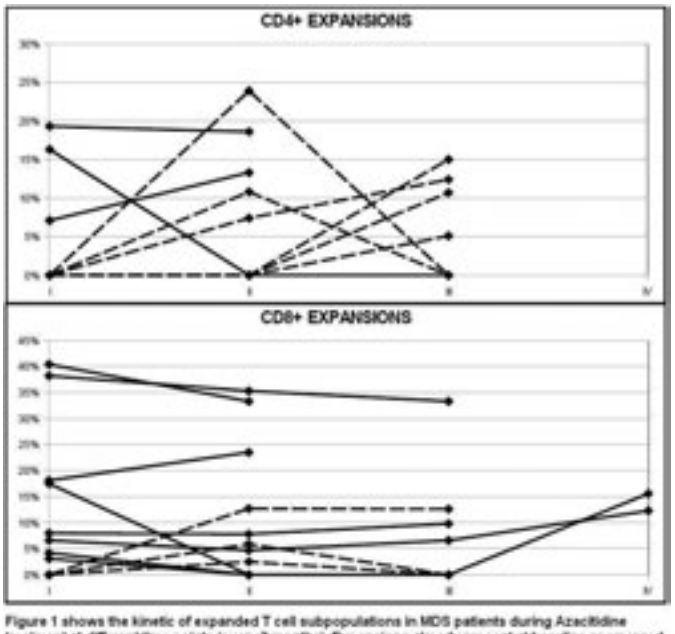

Figure 1 shows the kinetic of expanded T cell subpopulations in MDS patients during Azacitidine treatment at different time points (every 3 months). Expansions already present at baseline or emerged during therapy are represented as continuous or dashed lines respectively. By convention values below the mean + 3 standard deviations are represented with a value of 0.0 %.

PO-141

LOW RPS14 EXPRESSION NEGATIVELY CONDITIONS THE PROGNOSIS OF PATIENTS AFFECTED BY MYELODYSPLASTIC SYNDROMES WITHOUT 5Q- DURING AZACITINE TREATMENT

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Introduction. In the recent years, many efforts have been spent in order to understand the pathogenesis of the 5q- syndrome. The partial loss of function of the RPS14 gene, consequent to its haploinsufficiency, is now recognized as one of the principal causes of the selective apoptosis of the erythroid precursors. A multicentric Italian trial confirmed *in vivo* that RPS14 expression was low in 5q- cases and that its increased levels during treatment with lenalidomide well fitted with erithroid response. But low levels of RPS14 were measured also in MDS intermediate-1 patients without 5q- aberration, where low RPS14 levels defined a subgroup with a significant longer survival. In order to confirm these results, we analyzed the impact of RPS14 expression on outcome of 31 consecutive high-risk MDS patients treated with azacitidine at our center from 2007 to 2011. *Patients and Methods*. Bone marrow samples from 6 healthy donors (patients who underwent femur surgery) were used as controls; the stratification in 2 categories (RPS14 low and high) was defined according to the median value measured in the healthy donors (1.073 copies/104 copies of S18, used as internal control gene). Patients' characteristics (age, sex, IPSS, WPSS, blast count) did not differ between cases with high or low RPS14 levels. IPSS was intermediate-1 in 11 patients, and high in the remaining 20 (64,5%); at the start of treatment, WPSS was high or very high in 24 cases (77,4%). The remaining patients with intermediate-1 IPSS already received epoietin or G-CSF without any response. *Results.* For the entire series, 2-year overall survival (OS) was 63,2% and 2-year leukemia-free-survival (LFS) 53,7%. In univariate analysis, LFS was significantly affected by the quality of response after 24 weeks (P=0.001) and by the persistence of erythroid transfusion dependence at 24 weeks (P=0.03). OS was significantly affected by quality of response at 24 weeks of treatment (P=0.001) and by the erythroid transfusion dependence at 24 weeks (P=0.002). For patients with low RPS14 levels, 2-year LFS was significantly shorter, (39% for cases with low RPS14 versus 100%; P=0.018). Also the OS appeared negatively affected by low RPS14 levels (2-year OS of 51% for cases with low RPS14 expression versus 100% for cases with high levels), but without statistical significance (P=0.08). *Conclusions.* These results support the hypothesis that, if the RPS14 expression is really not conditioned by the methylation but only by the haploinsufficiency, the use of the hypomethylating agents in cases with low RPS14 levels could not be not so effective. On the contrary, these patients could be good candidates for alternative treatments, such as lenalidomide.

PO-142

GLOBAL DNA METHYLATION AS A PROGNOSTIC FACTOR FOR RESPONSE TO TREATMENT WITH 5-AZACITIDINE IN HIGH-RISK MYELODYSPLASTIC SYNDROMES

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Background. Among epigenetic processes, promoter DNA hypermethylation is now considered a common hallmark of cancer and the gene silencing induced by this phenomenon can be reversed. Myelodysplastic Syndromes (MDS) cells frequently show epigenetic abnormalities, such as aberrant hypermethylation of genes controlling cell proliferation or differentiation, which are usually associated with poor prognosis in MDS. That is why most of the current therapeutic approaches for MDS patients are based on epigenetic therapy. *Aims.* We investigated the association between global DNA methylation in MDS patients with clinical outcome. Results were compared to an age-matched control group of healthy subjects and to a group of *de novo* and secondary AML patients. Then we focused on the role of global DNA methylation to predict the clinical response to azacitidine. *Methods.* Bone marrow biopsy and mononuclear cells from 128 patients affected by MDS, comprising all subgroups, and 14 patients with high risk MDS treated with azacitidine were examined. Immunochemistry was performed on paraffin-embedded sections using anti-5-methylcytosine/5mc antibody. Double immunostainings were performed for nuclear 5-methylcytosine/5mc and one of four cytoplasmic/cell membrane markers: CD34 for precursors, MPO for myeloid cells, Glycophorin-C for erythroid cells, Factor VIII for megakaryocytes. LINE-1 sequences were measured by COBRA methodology and by Pyrosequencing technology. *Results.* Our results showed that in MDS the global DNA methylation was intermediate between normal controls and AML and significantly correlated with age, blast count and karyotype. Moreover we identified at multivariate analysis only global methylation and age as significant independent prognostic factors affecting overall survival (OS). When we evaluated the global DNA hypermethylation in patients treated with azacitidine, we observed that it decreased in 10 responder patients and anticipated the haematological response, whereas it didn't in 4 patients with stable disease. *Conclusions.* Our data confirmed that DNA hypermethylation in MDS correlates with the clinical outcome. Also, we demonstrated, not only that global DNA methylation could be used to monitor the effect of azacitidine, but also may anticipate the haematological response. A longer follow-up is needed to make any correlation with prognosis and overall survival.

PO-143

ROLE OF AUTOPHAGY IN MYELODYSPLASTYC SYNDROMES TO IMPROVE THERAPEUTIC EFFECT OF 5-AZACITIDINE

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Introduction. Myelodysplastic Syndromes (MDS) are clonal diseases characterised by ineffective hematopoiesis, peripheral cytopenias and variable risk of transformation to acute myeloid leukaemia (AML). Recently, 5-azacytidine (AZA) has been used to improve clinical outcomes, but despite an initial response obtained within first eight months of treatment, many patients progress to AML and die within two years from the therapy start. Our previous work suggested autophagy as a pro-survival compensatory pathway induced by long-term exposure to AZA in-vivo. *Methods.* In a training set of 19 MDS patients we evaluated proteomic changes after 4 or 8 months of standard treatment with AZA to identify by Reverse Phase Micro Array (RPMA) compensatory changes at proteomic level. In an additional set of 15 consecutive newly diagnosed MDS patients (IPSS int-2 or high) bone marrow cells have been exposed *in vitro* to AZA 1uM or 5uM for three days, then washed and treated with 1-2.5-5uM chloroquine (CQ, an autophagy inhibitor) for 12-24 hours to evaluate cell viability using a luminescence assay, changes in protein expression and cell-cycle by flow cytometry. Dose-response curve was generated for each drug and each condition, including pre-treatment and wash of each investigated drug; combination index was detected using the Chou`s method. *Results.* Autophagy was induced independently form the clinical response observed: ATG5, Beclin 1 and LC3B were significantly elevated after treatment (p values respectively <0.0001, 0.0056 and 0.0124), at downstream of mTOR, since mTORSer2448, AktSer473, AktThr308, ERKThr202Tyr204 (and in general proliferation markers) were not affected.After three days of 5uM AZA, CQ inhibited cell growth in a dosage and time-dependent manner, via G1 cell cycle arrest. Combination index of AZA and CQ was found to be >1, thereby indicating antagonistic drug interaction, sustained by AZA-mediated autophagy induction. *Conclusion.* Administration of CQ after AZA exposure is effective *in vitro* to inhibit cell growth in a dosage and time-dependent manner.

PO-144

TRADITION AND INNOVATION IN HEMATOLOGY: THE VIRTUAL MICROSCOPY

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Introduction. Medical images represent one of the most important diagnostic instruments in many specialties. Classical diagnostic hematological pathway concerns the identification and classification of cells derived from bone marrow and peripheral blood according to all international diagnostic protocols, a stakeholder step as well the first one in the integrated diagnostic process of haematological diseases. The current information and communication technology (ICT) era provides the opportunity to exchange, via internet, images and information without geographic limitation, saving time and resources. The computerized images standardized according to the significant content and the technological aspects, such as resolution, weight and compression, offer us today the highest excellence in terms of accreditation, training, and information exchange. All these systems and devices work with freezed images selected by the microphotographers. The develop of a new technology, the Virtual microscopy (VM) system, based on the scan of the whole smear open new and realistic opportunities: putting in the web the scan of the smears of PB and BM all the virtual community involved in this knowledge process can be trained adopting the same diagnostic procedures as for those adopted in the real life for the diagnosis at microscope of haematological patients. Images of reproducibly high quality are thus produced and shared within the workstation network, ready for analysis even while the next slides are still scanned. *Method.* We have evaluated the Zeiss Virtual Slide Scanner Metafer for a period of 2 months. This pilot project was focused on the evaluation of five Bone Marrow smears with a low proportion of blast count, scanned, stored and shared via net. The possibility to mark the blast cell coordinates represent a very strong and easy tool to discuss discrepancies to reach an harmonized consensus diagnosis. *Results.* This methodology represents the best tool to reach an harmonized morphological diagnosis among different groups for a consensual patient stratification. Moreover this system could be easily used for training and education in morphology in a web environment exactly reproducing the traditional microscope. *Conclusion*: VM has important future roles in accreditation and recertification. VM and digital analysis will continue to improve how morphology training and education is delivered.

Monoclonal Gammopathies

PO-145

SCLEROSTIN, PRODUCED BY MYELOMA PLASMA CELL, SUPPRESSES OSTEOBLAST ACTIVITY

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Introduction. Wingless-type (Wnt) signaling, through the secretion of Wnt inhibitors Dickkopf1 and soluble frizzled-related protein-2 and -3, plays a key role in the decreased osteoblast (OB) activity associated with multiple myeloma (MM)-bone disease. Based on the recent identification of sclerostin as another important negative regulator of bone formation, here we studied its involvement in the impaired bone formation occurring in MM-bone disease. *Methods.* CD138+ myeloma cells, isolated from bone marrow (BM) aspirates of 60 patients with newly diagnosed symptomatic MM, 38 MGUS controls, and 4 human MM cell lines (HMCLs) (namely H929, RPMI 8226, U266 and Karpas 929), were used for RNA or protein extraction, and co-culture experiments. BM stromal cells (BMSCs) were obtained from the adherent fraction of BM mononuclear cells, and used in co-culture experiments with HMCLs in the presence or in the absence of neutralizing anti-sclerostin antibodies. Subsequently, protein extraction from BMSCs was performed. *Results*. We demonstrated that sclerostin is expressed by HMCLs, and by plasma cells from bone marrow of the patients with MM-bone disease. Further, when co-cultured with HMCLs and in comparison with BMSCs alone, the BMSCs showed reduced expression of major osteoblastic markers such as collagen type I (COLL I), osteopontin, bone sialoprotein II (BSP II), and osteocalcin (OSTC) as well as decreased mineralized nodule formation and expression of activator protein 1-transcription factor (AP-1) family members including Fra-1, Fra-2 and Jun-D. In the same co-culture system, the addition of neutralizing anti-sclerostin antibodies at different concentrations (respectively, 50 and 500 ng/ml) progressively restored the OB functions. In addition, we showed that sclerostin also contributed to the upregulation of receptor activator of nuclear factor -B ligand (RANKL) and downregulation of osteoprotegerin (OPG). *Conclusions*. Our findings demonstrated the expression of sclerostin by CD138+ myeloma cells. They also highlighted the contribution of sclerostin to the development of MM-bone disease, that could be related to both the direct induction of OB suppression with impaired bone formation, and the indirect activation of osteoclasts bone resorption through the unbalanced RANKL/OPG ratio. Thus, sclerostin can provide a promising potential target for the development of novel therapeutics to rebuild bone mass in MM-bone disease.

PO-146

TARGETING NAD+ SALVAGE PATHWAY INDUCES AUTOPHAGY IN MULTIPLE MYELOMA CELLS VIA MTORC1 AND EXTRACELLULAR SIGNAL-REGULATED KINASE (ERK1/2) INHIBITION

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Background. intracellular nicotinamide adenine nucleotide (NAD+) is a coenzyme crucially involved in the regulation of several cellular processes. In mammals, NAD+ stores are continuously replenished by a salvage pathway from Nicotinamide through the rate-limiting enzyme Nicotinamide phosphoribosyltransferase (Nampt). Indeed, tumor cells exhibit aberrant metabolic activity characterized by high levels of aerobic glycolysis and increased turnover of NAD+ to support their rapid proliferation. In such scenario, promising results obtained in preclinical cancer models with Nampt inhibitors (such as FK866) suggest that Nampt activity represents an innovative therapeutic target for anticancer agents. *Methods.* a panel of 18 different MM cell lines, both sensitive and resist-

ant to conventional and novel anti-myeloma drugs and BM samples from MM patients were used in the study. The antitumor effect of FK866 was investigated by Annexin-V/propidium iodide staining, thymidine incorporation, Western-blotting, lentivirus-mediated shRNAs and gene expression profiling analysis. Intracellular NAD+ content was measured using a biochemical assay. Identification of autophagy was validated by 4 independent approaches: electron microscopy, proteolytic cleavage of endogenous LC3-I to LC3-II by Immunoblotting, formation of LC3 puncta pattern in GFP-LC3-transfected cells, as well as by gene expression profiling. Angiogenesis and osteoclastogenesis were measured *in vitro* using Matrigel capillary-like tube structure formation assay and osteoclast culture, respectively. In vivo study was performed using CB17-SCID mice xenografted subcutaneously with MM cells. *Results.* the chemical Nampt inhibitior FK866 triggered cytotoxicity in a panel of 18 MM cell lines and patient MM cells. Their viability was uniformly inhibited, with IC50 values at 96 hours ranging from 3-30nM. Additionally, Nampt inhibition killed, in a dose-dependent fashion, MM cells resistant to conventional and novel anti-MM therapies and overcome the protective effects of cytokines (IL-6, IGF-1), bone marrow stromal cells and primary osteoclasts. In contrast, FK866 treatment of PBMCs from 5 healthy volunteers did not affect cell viability. Nampt knockdown by RNAi confirmed its pivotal role in maintenance of both MM cell viability and intracellular NAD+ stores. Interestingly, cytotoxicity of FK866 triggered autophagy but not apoptosis. A transcriptional-dependent (TFEB) and -independent (PI3K/mTORC1) activation of autophagy mediated FK866 MM cytotoxicity. Finally, FK866 synergized with conventional and novel anti-MM therapies and demonstrated significant anti-MM activity in a xenograft-murine MM model, associated with down regulation of ERK1/2 phosphorylation and proteolitic cleavage of LC3 in tumor cells. Conclusion: In conclusion, we identify for the first time a link between intracellular NAD+ metabolism and autophagy in MM cells, providing the framework developing new targeted therapies in MM.

PO-147

NEUTROPHILS CD64 SURFACE EXPRESSION IS UP-REGULATED IN PATIENTS WITH MULTIPLE MYELOMA BY LENALIDOMIDE.

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Introduction. The high affinity Fcgamma receptor (Fc RI,CD64) is expressed almost exclusively on mononuclear phagocytes acting as cytotoxic trigger molecule in mediating several functions (phagocytosis, superoxide generation, cytokines release, immune complex clearance). Few Fc RI sites are normally found on polymorphonuclear leukocytes (PMNs) and increase after exposure to IFNy or G-CSF. PMNs express their Fc RI only when they are activated. While there is ample literature on the diagnostic utility of neutrophil CD64 as a biomarker for sepsis, little information is available regarding its immunomodulatory activity. Infection represent a significant cause of morbidity and mortality for patients with Multiple Myeloma (MM). The immune system abnormalities in MM, age-related comorbidities and antineoplastic therapies, are the main predisposing factors to infections. The most recently introduced anti-myeloma agents (lenalidomide, bortezomib) impact the immune system differently. Based on our observations and data from clinical trials, the infection rate in MM patients received lenalidomide is lower than other drugs and does not correlate with the grade of neutropenia. On the contrary, phagocytosis activity is strongly compromised in MM. *Methods.* We analyzed 30 patients with MM from Oct-2010 and Apr-2012 at our Institution. 20 patients in 1stline therapy (10 patients received a bortezomib- and 10 lenalidomide-based regimen) were followed up for at least 6 months. 10 patients affected by MGUS and 10 healthy adults served as controls. All the patients included in the study were monitoring for infections and a full inflammation status screening was performed. Cell surface expression of CD64, CD11b, CD16, and CD62L on peripheral blood neutrophils and monocytes was evaluated using the Epics XL-MCL flow cytometer and expressed as mean fluorescence intensities. PMNs and monocytes from 12 MM patients were also examined for phagocytic function using the Phagotest kit. Heparinized peripheral blood samples were incubated with opsonized FITC-labeled E.Coli. and than analyzed by flow cytometry. The phagocytic ability was expressed as percentage of fluorescent cells in the population studied and calculated by subtracting the percentage of the negative control sample (<1%) from the positive sample. *Results.* Neutrophils expressions of CD64 were significantly elevated in MM patients compared to MGUS group or healthy controls (P=0,01 and P= 0,007 respectively); there was no significant difference between the group of MGUS and healthy individuals (P= 0,94). The intensity of CD64 expression on neutrophils strongly increased during lenalidomide treatment (median±SD 20±12 vs 33±15, P=0,003). A similar enhancing effect was not seen with the bortezomib-based regimens. This marked CD64 upregulation by lenalidomide was not associated with modulation of CD16, CD11b or CD62L and was significantly correlated with dosage. PMNs of MM patients showed markedly decreased phagocytic capacity in comparison to MGUS and healthy individuals (P=0,0002 and P<0,0001). No significant rescue in the phagocytic PMN potential was seen during lenalidomide treatment (mean 40±15 vs 50±13, P=0,3). *Conclusion.* Presumably these findings indicate the marked CD64 upregulation by lenalidomide on the surface of MM PMN represents an early event in the activation process of leukocytes although is not sufficient in restoring phagocytosis.

PO-148

RELATIONSHIP BETWEEN BONE IMAGING FEATURES AND BONE MARROW CYTOKINE AND CHEMOKINE PROFILES IN PATIENTS WITH MONOCLONAL GAMMOPATHY

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Introduction. Osteolytic lesions are the hallmark of multiple myeloma (MM). In the last years new imaging technologies have been introduced in the evaluation of the skeleton such as magnetic resonance imaging (MRI) and positron emission tomography (PET)/computerized tomography (CT) scan improving the sensitivity in the detection of bone lesions. However the potential relationship between bone imaging features and the bone marrow (BM) cytokine and chemokine profiles is not known and has been investigated in this study. *Methods.* A cohort of 248 patients with monoclonal gammopathy (median age 71 years; range: 34-92 years) has been evaluated including 32 patients with MGUS, 36 patients with smoldering MM (SMM) and 180 patients with symptomatic MM (75% at the diagnosis and 25% at the relapse). The X-ray survey, vertebral MRI and total body PET/CT was performed in 172, 70 and 95 MM patients, respectively. The presence of the main cytogenetic abnormalities (hyperdiploid karyotype, del13, t(4;14), t(11;14), 17p-, amp1) has been investigated in purified CD138+ MM cells by FISH and the BM plasma was obtained from MGUS, SMM and MM patients. RANKL, OPG, IL-3, IL-7, DKK-1, Activin A, CCL3/MIP-1 , CCl20/MIP-3 BM plasma levels were measured by ELISA assay. Quantitative variables were compared by non-parametric Kruskal-Wallis and Mann-Whitney tests as appropriate and categorical variables were analyzed by Chi-square test. *Results.* We found that 59%, 86% and 63% of MM patients were positive at the X-ray survey, MRI and PET/CT, respectively. Approximately19% of MM patients with a negative X-ray were positive at the PET/CT, whereas 73% of those without osteolytic lesions were positive at the MRI for BM lesions with a prevalence of a focal pattern of infiltration. On the other hand 43% of MM patients negative at the PET/CT were positive at the MRI scan. Significant higher BM levels of all the cytokines and chemokines tested were demonstrated in MM and SMM as compared to MGUS ($p \leq 0.02$) and in symptomatic MM as compared to SMM and MGUS (P<0.02) with the exception of IL-3. A significant correlation between ISS staging system and the BM plasma levels of Activin A, CCL3, CCL20 and IL-7 (P<0.005) was demonstrated. Significant higher BM levels of CCL3, CCL20 and DKK-1 (P<0.05) were detected in MM patients with osteolytic lesions as compared to those negative at the skeletal X-ray survey. On the other hand, MM patients with a positive MRI scan have higher BM levels of Activin A, IL-3, CCL3, CCL20 and DKK-1 (P<0.05) as compared to those negative whereas MM patients positive at the PET/CT scan have significant higher levels of CCL3 and CCL20 (P<0.05) as compared to those negative Finally we found that BM plasma levels of DKK-1, Activin A, and CCL20/MIP-3 were significantly different in relation to the presence of the cytogenetic abnormalities including hyperdiploid karyotype, del13 and t(4;14). *Conclusions.* Our results identify the presence of a tight rela-

tionship between the BM cytokine and chemokine profiles and the bone imaging and molecular features in patients with monoclonal gammopathy.

PO-149

EFFICACY AND SAFETY OF BORTEZOMIB IN A CASE OF LIGHT-CHAIN DEPOSITION RENAL DISEASE (LCDD)

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Introduction. The Light chain deposition disease (LCDD) is characterized by the deposition of only one type of light chain in the renal tubular basement membranes. It can be associated to a plasma cell dyscrasia, however, it can occur in the absence of hematological disorder and then is defined idiopathic LCDD. We refer the case of a 65 years old woman hospitalized on march 2010 for a nephrotic syndrome characterized by hypertensive crisis, lower limbs edema, high 24 hours proteinuria(3 gr./day), severe anemia, absence of monoclonal component. The radiography of the skeleton showed no osteolytic lesion attributable to myeloma.The sampling of bone marrow had low cellularity with scanty infiltration of plasmacells (<5%) showing the following immunophenotype: CD38+/CD138+ 0.3%(k 49%, 39%),CD20-/CD27- 44.8%,CD20-/CD27+ 42%,CD19-/CD56- 60%,CD19-/CD56+ 11.4%, CD45+ 58%,CD117-/CD56- 67%. The renal biopsy confirmed the presence of PAS+ material,Red Congo neg., in mesangial compatible with kappa light chain deposition disease(LCDD). Serum Free Light Chains (sFLC)demonstrated an abnormal k/ ratio. Initially discharged from the Hospital with steroid therapy with poor results. Rehospitalized in June 2010 with acute respiratory failure complicating acute bilateral pneumonia.The bronchoscopy showed coinfection of Staph. Aureus and Pseudomonas aeruginosa. In September 2010 was performed an electromyography of lower limbs showing a damage consistent with steroid myopathy. *Methods.* We decided to start therapy with bortezomib and dexamethasone (according to Vel- Dex protocol: d1-d4-d8-d11 later amended to d1-d8-d15-d22) for a total of eight cycles, from September 2010 to September 2011. *Results.* During the second cycle we observed the rapid regression of the nephrotic syndrome. The only toxicity, of grade II, was iatrogenic neuropathy improved after switching to weekly administration of bortezomib. At the clinical revaluation there was a complete regression of all symptoms present before the start of therapy. The sFLC had a normal kappa/lambda ratio.The renal evaluation showed a creatinine clearance of 69 ml/min measured by Cockcroft-Gault with a residual proteinuria 9.3 mg /L (n.v. 0-45). *Conclusions.* We believe that therapy with bortezomib is safe and effective. However the rarity of this disease makes it difficult to realize prospective studies to assess the prognosis in these patients.

PO-150

A FRAIL ELDERLY PATIENT WITH MULTIPLE MYELOMA AND MYELODYSPLASTIC SYNDROME: ACHIEVEMENT OF A STABLE DISEASE AND HEMATOLOGIC IMPROVEMENT WITH DEFERASIROX

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Background Multiple Myeloma (MM) and Myelodysplastic syndromes (MDS) tipically affect the elderly. Deferasirox is an oral iron chelator, aimed at preventing organ damage due to transfusional iron overload; inhibition of NFkB seems to be another pharmacological effect of the drug. We describe a frail patient with MDS and MM who achieved hematologic improvement and a stable disease under deferasirox treatment. Case Report: A 79 year-old female patient was diagnosed as having refractory anemia (RA) in March 2010, karyotype 46,XX (low IPSS and WPSS); Hb level at diagnosis was 7.2g/dl, with serum erythropoietin levels of 705 mIU/mL. Concomitant MGUS IgG K was observed. Median transfusion requirement was 2 RBC units/month; in June 2010 serum ferritin (SF) was 2718 ng/dL, so she started deferasirox treatment at the dose of 10mg/kg/day. In August 2010 a massive pulmonary thromboembolism occurred and patient was hospitalized: in that occasion bone marrow aspiration documented an evolution from MGUS to MM (marrow plasma cells 22%, ISS II). Monoclonal component (MC) was 2.01 g/dL. Lytic bone lesions were observed, so that monthly zoledronic acid was started. She was initially treated with melphalan-prednisone regimen, promptly interrupted because anemia dramatically worsened (Hb 5.2g/dL); considering the frailty of the patient, we continued only with dexamethasone 20 mg weekly. Transfusion requirement significantly increased up to 4 RBC units/month; therefore in October 2010 deferasirox dose was increased to 20 mg/kg/day. Subsequent follow-up was unremarkable and, unexpectedly, her transfusion need decreased to 1 RBC unit/month, achieving an Hb level of 10 g/dl in April 2011. SF was 2310 ng/dL; bone marrow aspirate documented a percentage of 20% of plasma cells, with no progression on lytic lesions. Interestingly, in the following months we also observed a slow decrease of MC, reaching a value of 1.20 g/dL in November 2011. Transfusion requirement, SF and Hb levels remained nearly stable. According to International Working Group (IWG) 2006 criteria, we can assume that the patient has obtained an erythroid response regarding her MDS features. Concerning her MM, patient maintained a stable disease. Discussion: Our patient achieved an unusual long term stabilization of MM without a conventional antineoplastic treatment, except for the low dose of dexamethasone. This observation led us to hypothesize a dual therapeutic action of deferasirox on MDS cells and on MM cells: in fact, a recent *in vitro* study showed that deferasirox is a potent inhibitor of NFkB, which is abnormally activated in MDS blast. Interestingly, NFkB is also the target of the antimyeloma activity of the proteasome inhibitor bortezomib, leading to the downregulation of angiogenesis factors, cytokine signalling and MM cell survival. In conclusion, it is appealing to suppose in our patient a double role of deferasirox-mediated NFkB inhibition, both on MDS cells and on MM cells.

PO-151

BORTEZOMIB, NON-PEGYLATED LIPOSOMAL DOXORUBICIN AND DEXAMETHASON (PAD REGIMEN) IN RELAPSED OR REFRACTORY MULTIPLE MYELOMA: RESULTS FROM A PROSPECTIVE STUDY

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Introduction. Combining bortezomib with pegylated liposomal doxorubicin for the treatment of Multiple Myeloma (MM) may reciprocally increase their efficacy *in vitro* and appears to offer higher overall response rates *in vivo*. With the aim to verify the synergistic interaction towards myeloma cells we planned a prospective study with bortezomib, non-pegylated liposomal doxorubicin and dexamethason in patients with relapsed/refractory multiple myeloma (R/R MM). In this setting of patients, showing a poor outcome because of multi-drug resistance, low-performance status and toxicity to previous chemotherapy, bortezomib, by inhibiting proteasome function, may enhance chemosensitivity to doxorubicin and overcome drug-resistance. Thus, to improve outcome minimizing therapy-related toxicity, bortezomib was added to non-pegylated liposomal doxorubicin and dexamethason (PAD regimen). Patients and Methods. From November 2005 and January 2012, 50 patients with R/R MM (relapsed n= 37 and refractory n= 13) referred to the our Institution received PAD regimen. Male/Female ratio: 27/23; Median age: 61 years (range 34-79); median time from diagnosis: 32 months (range 4-121); median of previous therapy lines: n= 3 (range 1-5); patients previously underwent to autologous and allogeneic hemopoietic stem cell transplantation (auto- and allo-HSCT): 22 and 5, respectively. Planned treatment: bortezomib 1,3 mg/mq iv days 1,4,8,11; non-pegylated liposomal doxorubicin 30 mg/mq on day 1 and dexamethason 40 mg days 1-4 of a 28-day cycle up to 6 cycles. Results. Forty patients (80%) received the planned treatment schedule whereas 10 patients did not complete it because of toxicity (1 patients) and resistant or progressive disease (9 patients). Median time to best response was 3 months (range 2-6). The overall response rate was 74% with 10 CR (20%), 15 vGPR (30%) and 12 PR (24%). Fifteen of the responder patients underwent HSCT (auto-HSCT: 9; allo-HSCT: 6). The previous use of anthracyclines (35 patients) and bortezomib (3 patients) did not seem influence the response. Median duration of response was 24 months (range 6-57 months). After a median follow-up of 58 months, 14 (28%) patients were alive and, of these, 7 (14%) in Continue CR. The safety profile was manageable: the hematologic grade 3/4 toxicity (neutropenia, thrombocytopenia and anemia) was 28%; non-hematologic

grade 3/4 toxicity (sensory or motor neuropathy, infections, fever, fatigue, diarrhea) was 36%. Despite heavy previous treatments, including anthracycline-based, no significant cardiac toxicity was observed. Conclusion. According to our study, the PAD regimen appears feasible and effective in both elderly and heavily pre-treated R/R MM patients. In fact a significant improved clinical outcome in our cohort of patients was observed.

PO-152

INFECTIOUS COMPLICATIONS IN ELDERLY PATIENTS WITH MULTIPLE MYELOMA: A COMPARISON BETWEEN "BORTEZOMIB INCLUDING" AUTOLOGOUS STEM CELL TRANSPLANTATION AND "CONVENTIONAL" AUTOLOGOUS STEM CELL TRANSPLANTATION

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Introduction. Autologous Stem Cell Transplantation (ASCT) is still a mainstream strategy in Multiple Myeloma (MM) patients under 60 years. However also fit elderly patients can benefit from ASCT including strategies. CY-BOR is a schedule currently used in our centre in fit patients >60 years, combining bortezomib (BOR), cyclophosphamide (CY) and dexamethasone (DEX); this schedule has been incorporated in a global strategy, in the context of a clinical prospective multicenter study, including induction and mobilizing therapy with CY-BOR, followed by ASCT with BOR-High-Dose-Melphalan (HD-MEL). As the use of BOR is associated with an increased risk of viral infections, we decided to retrospectively compare data concerning the engraftment (in terms of WBC and PLT take) and infectious complications after ASCT in two groups of patients, treated according to either CY-BOR protocol or other conventional therapies, followed by ASCT with HD-MEL without BOR. *Methods.* the first group includes 23 patients (11M/12F), median age 65 years (range 60-75), who received three courses of CY-BOR as induction therapy, were mobilized with CY 3 g/m^2 (at day +8) and received ASCT with MEL (140-200 mg/m^2 day -1) and BOR (1mg/m2 days -6, -3, +1, +4). The second group includes 16 patients (12M/4F), median age 65 years (range 60-73), who were treated with different induction schedules not including BOR, were mobilized with CY (3-7g/m^2) and underwent ASCT with MEL (140-200 mg/m^2) without BOR. *Results.* in the first group a median number of 5.1x10^6 CD34+ cells/kg (range 2.2-7) were infused. Median time for PMN engraftment was 11 days (range 10-14) and 14 days(range 11-24) for PLT>=20.000/mcl. We observed FUO in 6 patients, a sepsis caused by streptococcus bovis in 1 patient, pneumonia in 3 patients, asymptomatic CMV reactivation in 1 patient, CMV related pneumonia in 1 patient. In 11 patients (48%) we did not observe neutropenic fever. In the second group a median number of 3.79x10e6 CD34+ cells/kg (range 2.3-7.3) were infused. Median time for PMN engraftment was 11 days (range 9-13) and 12 days (range 10-16) for PLT>=20.000/mcl. We observed FUO in 3 patients, pneumonia in 2 patients. In 11 patients (69%) we did not observe any infections. *Conclusions.* We did not observe any differences in engraftment time between the two groups. Patients treated according to the CY-BOR protocol experienced a slightly higher number of infectious complications. Although we cannot draw any statistical correlation due to the small sample, we can argue that BOR might induce a deeper immune suppression and consequently expose patients to a higher number of infectious complications.

PO-153

CD117 (C-KIT) AND CD43 EXPRESSION AND CLINICAL OUTCOME IN PATIENTS AFFECTED BY MULTIPLE MYELOMA (MM)

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Background. The survival of patients affected by MM is variable depending upon the tumour mass at the diagnosis and by the intrinsic biological characteristics of tumour cells. Flow cytometry and immunological methods have allowed the characterization of a series of surface antigenic molecules expressed on either MM or normal cells. With this technique several molecules differentially expressed on normal and MM cells and correlated with the prognosis of MM patients have been identified. In details B-associated antigens, growth factor receptors, myeloid antigens and adhesion molecules can be found on pathological plasma cells. At this regard some studies have demonstrated that in about 50% of MGUS patients and 33% of MM patients the plasma cells express CD117 (c-kit), while normal plasma cells are CD117 negative. Moreover, both the normal plasma cells and those of patients with MGUS are usually positive for the CD43. *Methods.* We have analyzed the bone marrow blood of 57 patients affected by MM. 35 out of 57 presented a IgG component and the remaining 22 patients were IgA. On the basis of the staging criteria (Durie e Salmon), 29/57 pts. were in stage II and 28/57 in stage III; the clinical stage (remission, progression or stable disease) was defined with clinical re-evaluation after chemotherapy and/or restaging at 6 months from diagnosis. *Results.* The immunophenotype of bone marrow plasma cells demonstrated the expression of CD38 (very bright) and of CD138 while CD19 was absent; 44/57 were CD43+(dim) and 15/57 CD117+(dim). 13 out of 15 CD117-positive patients showed a specific immunophenotypic pattern (CD117+/CD43-). These patients were in stage II and showed a favorable clinical outcome, as demonstrated by a higher DFS and OS than the remaining patients. *Conclusions.* The possible prognostic role of CD117 and CD43 in MM warrants further clinical investigation on a larger series of patients even on the basis of new therapeutic strategies.

PO-154

GENOMIC CHARACTERIZATION OF BOTH CD138+ NEOPLASTIC CLONE AND CD138- MEMORY B CELLS IN NEWLY DIAGNOSED MULTIPLE MYELOMA PATIENTS BY SNP ARRAY 6.0

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Background. Although the advances in Multiple Myeloma (MM) therapy, the disease remains incurable. The existence of Myeloma Propagating Cells (MPCs) is considered one of the major causes of MM drug-resistance, leading to relapse. However, very little is known about the molecular characteristics of MPCs, even if some studies suggested that these cells have phenotypic characteristics resembling the memory B cells. *Methods.* We collected the CD138+ and CD138-19+27+ cell fractions from bone marrow (BM) and peripheral blood (PBL) of 44 newly diagnosed patients (pts), 3 MGUS pts and 8 relapsed pts. For each pts, we performed a flow-citometric characterization of the enriched cell fractions. The complete set of genomic aberrations was evaluated by SNP Array 6.0 in one selected pts for each cell population fractions and copy number analysis was performed with Genotyping Console software. *Aim.* To molecularly characterize the CD138+ neoplastic clone and the memory B cells located both in BM and in PBL. *Results.* Both BM and PBL CD138+ cell fractions presented the same genomic macroalterations in chromosome (chr) 1, 2, 6, 7, 10, 11, 12, 13 and 14. There were eterozygous macrodeletions in chr 1p (44Mb), 2p (2 Mb), 6q (36 Mb), 10q (1,5 Mb), 11p (7 Mb), 12p (21 Mb) and 12q (8 Mb); in chr 7p, 13q and 14q the deletion is extended in the whole arm. We founded a unique amplification in the long arm of chr 1 (copy number state: 3). In the BM and PBL CD138-19+27+ cell fractions any macroalteration was highlighted; on the contrary, several microalterations (range: 1-834 Kb) unique of the memory B cells clone, were highlighted. Both in CD138-19+27+ cell fractions from BM and PBL we detected microalterations that were out of regions of genomic variants and possibly associated with the MM disease. Three focal microdeletions were shared by both memory B cells clone and the genes involved are SKT, CES1P1, MIR650. In the memory B cells clone circulating in PBL we also detected six focused microdeletions and the genes involved are HMGCLL1, DLGAP2. In the memory B cells clone homing in BM we observed ten microdeletions and one microamplification and the genes involved are RCOR3, PRR16, TSC1, ETS1, RBFOX1. These genes are of relevant importance for their involvement in cholesterol metabolism, embryonal development and transcriptional regulation. *Conclusions.* Our preliminary data suggested that the neoplastic clone CD138+ resume the end of a complex process of a tumorigenesis, proven by the presence of numerous macroalterations, probably due to an estabilished genomic instability. In contrast, the memory B cells lack these macroalterations but have some interesting microalterations, supporting the idea that

these post-germinal centre cells are involved in the trasforming event that originate the neoplastic clone. Results need to be confirmed in a higher number of pts, in order to get more insights into the role of involved genes in the tumorigenesis of MM.

PO-155

MYXOVIRUS RESISTANT PROTEIN 1 (MXA) EXPRESSION IN MULTIPLE MYELOMA CELLS

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Myxovirus resistant protein 1 (MxA) is a type I interferon (I-IFN)-inducible GTPase with antiviral activity. Its expression is driven by Interferon Stimulated Genes (ISGs) in response to viral pathogens. I-IFN plays a controversial role in multiple myeloma (MM) and used as maintenance therapy with the goal of reducing progression free survival. Considering that ISGs expression phenotype, represented by the MxA protein has been shown in cancer cell lines but not in their normal counterparts we test MxA expression on neoplastic and normal plasma cells. Preliminarily MxA was studied by western blot analysis on U266, RPMI 8266 and KMS-11 multiple myeloma cell lines. We then analyzed the *in vivo* expression of the same marker on bone marrow biopsies by using immunohistochemistry. Anti-MxA and anti-CD138 were applied on decalcified sections obtained from 14 MM cases and 6 control(CTR) . Cases were evaluated by two pathologists and scored as percentage of MxA+ neoplastic cells (10-50% - point 1, between 50 and 90% - point 2, >90% point 3) and intensity of the staining (weak - 1, moderate - 2 or strong - 3) was assessed for each case. Plasmocytoid Dendritic Cell (pDC) infiltration in bone marrow was also evaluated. By postulating a clinical significance of the tumor heterogeneity in MxA expression we correlated the MxA score (sum of expression and intensity) with clinical response (complete remission -CR- or very good partial remission - VGPR and progression free survival -PFS) using SPSS 17.0. All the patients received thalidomide and dexametazone as induction therapy, and 6 of them underwent bortezomib therapy. After induction all the patients underwent stem cell collection and double autologous transplantation conditioned with high dose melphalan. Western blot analysis revealed MxA expression on multiple myeloma cell lines. Whereas no reactivity was found in normal plasma cells from CTR, neoplastic cells expressed MxA in all cases (14/14 MM): MxA was expressed in more than 90% of plasma cells in 8 patients, more than 50% in 3 and less than 50% in 3. Intensity of expression was strong in 5 patients, variable in 5 and weak in 4 patients. pDC infiltration was clustered in 6 patients, rare in 4 and moderate in 3, not evaluable in one. None of the patients had active viral disease at diagnosis. Response to treatment was CR or VGPR in 11 cases, 7 progressed or relapsed and one patient died due to progression (median follow-up 55 months). On 5 patients with highest MxA score (6) only 1 relapsed (20%, OR 3.33), whereas 6 out 9 with lower scores relapsed (66%). pDC marrow infiltration seems to be not associated with response. Despite the low number of bone marrow specimens examined, this is the first demonstration that the dualism of phenotype based on the expression of MxA is present in multiple myeloma and that this is confirmed *in vivo*. The interaction between pDC and MM cells, the role of this interaction on MxA expression and the prognostic relevance of this phenotypic dualism should be further studied.

PO-156

MULTIPLE MYELOMA AND MONOCLONAL GAMMOPATHY OF UNDETERMINED SIGNIFICANCE:CYTOKINES PATTERN AND GENE EXPRESSION OF WT1

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Introduction. Multiple myeloma(MM) is a plasma cell dyscrasia characterized by proliferation of malignant plasma cell in the bone marrow (BM). It's usually evolves from an asymptomatic premalignant stage of clonal plasma cell proliferation termed "monoclonal gammopathy of undetermined significance"(MGUS). The bone marrow microenvironment (BM) is an important factor for progression disease from MGUS to MM because produces angiogenic factors which allow MM cells to proliferate and resist the induction of apoptosis.On the other hand myeloma cells directly produce several pro-angiogenic molecules and more cytokines wich induce their expression in bone marrow stromal cells. This in turn stimulates myeloma cell growth in a paracrine fashion.Recently study suggest that in MM patients with unfavorable prognosis the increased BM angiogenesis is due either to the aberrant expression of angiogenic genes suc as Wt1. Our Objective was to evaluate sieric and plasmatic concentrations of IL-6, TNF-a, VEGF in MM and MGUS patients and evaluate Wt1 expression gene in a part of our patients (MM-MGUS-controls). *Matherials and Methods*. We had analyzed sieric and plasmatic samples and clinic and laboratoristic characteristics of 44 MM patients(5 of them with MM smoldering), 48 MGUS patients and 14 health controls from 2004 to now. We made blood peripheral samples,we have separated serum from plasma to perform TNF-a, IL-6, VEGF dosage with ELISA test.For Wt1 dosage we have separated mononucleated cells with RT- PCR.Statistical evauation was performed using t-test (paired test and unpaired test) and Spearman test. P≤0.05 was designated statistically significant. *Results.* Statistical analysis revealed no significant differences between MM and MGUS patients regard sieric and plasmatic concentrations of TNF-a and IL-6.Only difference statistically significant between MM and MGUS patients was found in VEGF sieric concentration(P=0.012). IL-6 levels was found progressively high in three groups (MM>MGUS>controls).Only in MM patients we demostrated statistically significant differences between sieric and plasmatic concentrations of VEGF (P<0.001),TNF-a (P<0.02) and IL-6(P<0.005). Also significant relationship was found between VEGF and IL-6 and blood platelets number.Wt1 dosage was revealed inferior limit of sensibility in all three groups of patients. *Conclusions.* In conclusion the evolution from MGUS to MM is still in course of study but certainly BM microenviroment play an important role for progression disease.It's sigificant to consider how many things are in common between MM and MGUS microenviroment in order to better identify prematurely who,among MGUS patients, will evolve to MM. The role of these cytokines remain unclear but in our study significant different of serum VEGF between MM and MGUS patients and progression increase of IL-6 from MGUS to MM induce towards other studies to identify a group of MGUS patients with high risk progression disease.

PO-157

A RETROSPECTIVE ANALYSIS ON THE IMPACT OF PROGNOSTIC FACTORS IN PREDICTING EVOLUTION TO SYMPTOMATIC MULTIPLE MYELOMA IN 397 SMOLDERING MULTIPLE MYELOMA (SMM) CASES: THE EXPERIENCE OF GIMEMA MULTIPLE MYELOMA LATIUM GROUP

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Introduction. SMM is an asymptomatic plasma-cell proliferative disorder associated with a high risk of progression to symptomatic multiple myeloma (sy-MM). Patients with SMM meet the diagnostic criteria of MM [serum monoclonal component (MC) higher than 3 g/L and a proportion of bone marrow plasma cells (BMPC) ≥10%] in the absence of clinical manifestations. Prognostic factors for the progression and outcome of this disease are unclear. In this retrospective study, we have analyzed some predictors of development in sy-MM in SMM. Moreover, In some of these SMM patients, we also had the opportunity to compare the risk of progression predicted by the % of bone marrow plasma cell (BMPC) involvement observed in the bone marrow biopsies (BMB) versus that observed in bone marrow aspirates (BMA). METHODS For this study, 397 patients with SMM observed in 12 centers of the Multiple Myeloma GIMEMA Latium Working Group between 01/1980 and 07/2010 were analyzed. At progression to sy-MM, the severity of clinical presentation was graded according to the need of intensive supportive care. RESULTS After a median follow-up of 135 months, the cumulative incidence of progression (CIP) rates to sy-MM were 45%, 55% and 75% at 10, 15 and 20 years, respectively. Hb ≤12.5 gr/dL, MC 2.5 gr/dL and BMPC 60% were the only parameters negatively affecting the CIP. In particular, 7/397 (1.7%) patients with BMPC 60% had a 5.6-fold increased risk of fast progression (within 2 years) to sy-MM. This pro-

gression occurred with severe clinical manifestations in 62% of cases. BMB was more sensitive for the detection of BMPC involvement, even though BMA was a more reliable indicator of a rapid progression to sy-MM. *Conclusions.* These data show that SMM patients with 60% BMPC rate at diagnosis present a very rapid progressions to sy-MM characterized, in the majority of cases, by more severe clinical manifestations and suggest that these patients should probably be candidate to standard treatment soon at diagnosis. Moreover, BMA is more sensitive than BMB in the identification of these very high risk SMM patients.

PO-158

THE BCL-2 PROTEIN FAMILY IN THE PROGRESSION OF MULTIPLE MYELOMA: CONSTITUTIVE EXPRESSION, PROAPOPTOTIC EFFECTS OF THE BCL-2/BCL-XL INHIBITOR ABT-737 AND CLINICAL CORRELATIONS

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Introduction. Monoclonal plasma cells disorders (PCD) are clinically heterogeneous, including monoclonal gammopathy of undetermined significant (MGUS), smoldering multiple myeloma (SMM), symptomatic MM and plasma cell leukemia (PCL). Several molecular characteristics have been described in PCD, including the aberrant expression of the Bcl-2 protein family, which prompt innovative therapeutic approaches based on the Bcl-2 targeted inhibition. However, neither the expression pattern of the Bcl-2 protein family, nor differences in the sensitivity to specific inhibitors have been associated with the different clinical statuses. *Aims*. We therefore examined the constitutive expression of the Bcl-2 protein family (Bcl-2, Bcl-xL and Mcl-1) and the functional and molecular effects of the Bcl-2/Bcl-xL inhibitor ABT-737 (kindly provided by Abbott Laboratories) on purified primary CD138+ cells from 11 SMM, 20 symptomatic MM (8 at diagnosis and 12 at relapse) and 4 PCL samples. *Methods.* Expression of the Bcl-2 protein family was evaluated by western blot analysis. The cytotoxicity of ABT-737 on MM cells was established using the MTT assay. The drug concentration inducing 50% cell killing (IC-50) was calculated from the dose-response curve. Cell cycle inhibition and induction of apoptosis were analyzed by flow cytometry using the Acridine-Orange (AO) technique and by Annexin V binding assay. *Results.* Our data indicate that the constitutive protein expression of Mcl-1 is significantly higher (P=0.059) in MM samples at diagnosis compared to SMM (mean ratio Mcl-1/GAPDH: 1.70±0.51 vs 0.95±0.27) and slightly decreases in the more aggressive PCD disorders (1.1±0.21 and 1.21±0.83 in relapsed MM and PCL, respectively). Bcl-2 and Bcl-xL did not differ among samples from these different groups of patients. A marked ABT-737-induced pro-apoptotic activity was observed on purified CD138+ cells from primary samples. However, this effect was significantly (P=0.02) higher on CD138+ cells from SMM and on newly diagnosed MM compared to relapsed MM and PCL samples, which proved progressively less sensitive to ABT-737. In particular, the pro-apoptotic activity of ABT-737 gradually decreased from SMM, to newly diagnosed MM, relapsed MM and PCL, with a net apoptosis (sub-G0/1 peak) at 24 hours in the presence of 1000 nM ABT-737 of 55.9±15.2%, 49.3±21.3%, 37.6±10.6% and 33.3±17.9%, respectively. *Conclusions.* In summary, these data show that Mcl-1 is overexpressed in PCD, increasing from SMM to MM. Nevertheless, the activity of the Bcl-2 inhibitor ABT-737 is preserved, showing that in MM cells the interaction between Mcl-1 and other pro-apoptotic members, such as Bim, plays a major role in the response to ABT-737. Moreover, the highest levels of sensitivity of SMM to ABT-737, associated with the lowest levels of Mcl-1 expression, prompt new investigational approaches for the management of patients with SMM.

PO-159

CORRELATION BETWEEN LOW LEVEL OF VITAMIN D AND MULTIPLE MYELOMA

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Introduction. Vitamin D (25(OH)d) is a fundamental mediator of skeletal metabolism due to its ability to stimulate the absorption of calcium and phosphate through the intestinal mucosa and promote bone mineralization. Many reports suggest that vitamin D induces cellular differentiation, inhibits proliferation and angiogenesis and plays a role in promoting apoptosis, so an inverse correlation between 25(OH) levels and cancer risk in some solid tumors have been observed. In multiple myeloma cell lines anti proliferative and pro-apoptotic effect of vitamin D it's been reported. Low levels of vitamin D has been associated with higher ISS at diagnosis in multiple myeloma patients. *Methods.* From October 2011 we collected serum of 10 consecutive patients with newly diagnosis of myeloma (8 males, 2 females, mean age 62 years, range 40-74) in order to asses vitamin D levels and its correlation between ISS stage and CRAB criterias of treatment. Vitamin D levels were determinated using vitamin D total assay from Roche Diagnostics on a cobas C6000 platform and deficiency of vitamin D were defined as levels lower than 20 ng/ml. *Results.* According to Durie and Salmon staging 5 patients were stage I, 3 patients were stage II and 2 patients were stage III. 8/10 patients has vitamin D deficiency, with a mean concentration of vitamin D of 16 ng/mL (range 7-20 ng/dL) . The 2 patients without deficiency of vitamin D were both in stage III and ISS II and both fill at least 1 CRAB criteria's for treatment, while the 8 patient with deficiency were 5 in stage I, 2 in stage II and 1 in stage III and the same correlation between Durie and Salmon stage and ISS. In these 8 patients only 2 has CRAB criteria for therapy. *Conclusions.* Even if in small numbers no correlation between low levels of vitamin D and worse clinical and prognostic presentation of myeloma was shown. By contrast the worse presentation was in the only 2 patients who showed normal levels of vitamin D. What is of interest is that the proportion of carential patients is very high and is higher that what expected in normal population, suggesting a role of low levels of vitamin D in the onset of multiple myeloma. The study is going on and larger cohort is needed to confirm this preliminary observation and we are now analizing if levels of vitamin D could be implicated in the response to therapy in multiple myeloma patients.

PO-160

CHROMOSOME 1 ABNORMALITIES AND SURFACE CD19 EXPRESSION PREDICT POOR OVERALL SURVIVAL IN ELDERLY NEWLY DIAGNOSED MULTIPLE MYELOMA (MM) PATIENTS, ENROLLED IN THE GIMEMA-MM-03-05 RANDOMIZED CONTROLLED TRIAL

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Introduction. Chromosome 1 abnormalities (1p deletion and 1q amplification) have been analyzed in MM by several Authors and, at present, no uniform consensus has been obtained, concerning their clinical impact. The aim of this study is the evaluation of the prognostic impact of chromosome 1 abnormalities and immunophenotypic features in 511 newly diagnosed MM patients, older than 65 years, randomly assigned to receive either Bortezomib-Melphalan-Prednisone-Thalidomide followed by maintenance with Bortezomib-Thalidomide (VMPT-VT, N=254) or Bortezomib-Melphalan-Prednisone (VMP, N=257). *Methods*. Samples were suitable for FISH analysis in 336/511 patients (66%). FISH was performed on BMPC purified using anti-CD138-coated magnetic beads. Nuclei from fixed PC were prepared for interphase FISH using standard methods. DNA probes were used to detect 13q14, 1p36, and 17p13.1 deletions, 1qter amplification; t(4;14)(p16;q32), t(14;16)(q32;q23), t(11;14)(q13;q32). The immunological phenotype of bone marrow plasma cells (BMPC) was assessed in 399 patients using quadruple combinations of MoAbs for the detection of the following

antigens: CD38, CD138, CD56, CD45, CD40, CD19, CD20, CD52, CD117, kappa/lambda. *Results.* Frequency of deletion of chromosome 13q14 and 17p13.1 and IgH translocations, and the absence of significant prognostic relevance on PFS has been previously reported by Palumbo et al (JCO 2010). Chromosome 1 abnormalities were evaluated in 278 unselected patients. 1q amplification (amp1q) was observed in 130 patients (47%) and 1p deletion (del1p) was present in 24 patients (8.6%). Median follow-up was 42 months (range 1-69). The overall median PFS was 32 months. Median PFS was 29.7 months in amp1q-positive and 34.1 in amp1q-negative (P=0.219), while it was 24.6 months in del1p-positive and 33.2 in del1p-negative group (P=0.03). In all patients, median OS was not reached: the 4-year OS was 74%. The 4-year OS was 68.5% in amp1q-positive vs 78.9% in amp1q-negative group, (P=0.04) and it was 58.3% in del1p-positive vs 75.5% in del1p-negative group (P=0.02). In patients carrying at least one aberration of chromosome 1 (amp1q and/or del1p), the shorter OS was even more significant (P=0.005). Preliminary analysis of immunophenotipic data emphasized the shorter OS of CD19 positive patients (P=0.013). In a multivariate Cox regression model, independent predictors of shorter OS were chromosome 1 aberrations (HR, 1.95; 95% CI, 1.21 to 3.16; P=0.006), CD19 expression (HR, 1.99; 95% CI, 1.15 to 3.45; P=0.015), ISS stage II vs I (HR, 1.73; 95% CI, 1.02 to 2.93; P=0.04) and ISS stage III vs I (HR, 2.46; 95% CI, 1.40 to 4.35; P=0.002), regardless of treatment. *Conclusions.* Chromosome 1 abnormalities and CD19 are independent poor prognostic factors for OS in MM patients treated with novel Bortezomib-based regimens. A longer follow-up is needed to confirm the significant relevance of these results.

PO-161

NEUROLOGICAL MONITORING DURING BORTEZOMIB AND/OR IMIDS REDUCED THE DEVELOPMENT OF SEVERE PERIPHERAL NEUROPATHY IN MULTIPLE MYELOMA PATIENTS: A SINGLE CENTER EXPERIENCE

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Introduction. Dose-limiting peripheral neuropathy (PN) has been reported up to 75% of multiple myeloma (MM) patients (pts) treated with Bortezomib and/or IMIDs, requiring dose reduction, delay or interruption. Careful neurological monitoring and early diagnosis of PN may prevent irreversible neurological damage. We performed a monocentric prospective study to investigate the possible benefit of regular neurological assessment in reducing the development of severe PN. *Methods.* All patients were evaluated before, during and after MM treatment by clinical and neurophysiologic assessment. The neuropathy was graded in levels 1 to 4 according to the National Cancer Institute–Common Toxicity Criteria version 3 [NCI-CTC]. Neuropathic pain intensity was expressed according to the patient- reported Numerical Rating Scale [NRS]. *Results.* Between March 2007 and June 2011, 100 consecutive pts were included. Clinical characteristics were: median age 59 (32-77); 46 men and 54 women; Durie and Salmon stage I-II and III were 42 and 58 pts, respectively. Seventy five pts received > 2 lines (range 1-7). Sixty-three pts were treated with bortezomib, 31 with thalidomide, 25 with bortezomib-thalidomide combination, 51 with lenalidomide. After a median time of 22 months (range 1-171) from the start of treatment 65 pts developed PN (56 sensitive, 9 sensitive-motory), 31 during the first line therapy, 29 during the second one and 5 after the second line of therapy. At the time of PN diagnosis 26 pts were receiving bortezomib, 15 thalidomide, 20 bortezomib-thalidomide association, 4 lenalidomide. In 2 cases PN was diagnosed before starting the treatment. The median time to PN diagnosis was 2.8 months for bortezomib, 10.5 months for thalidomide, 2 months for bortezomib-thalidomide. There was no significant correlation between PN occurrence and cumulative dose of bortezomib (median 30 mg) and thalidomide (median 9.900 mg). According to the NCI-CTC toxicity criteria: 25 developed grade 1, 34 grade 2, 6 grade 3, 0 grade 4 PN. Neuropathic pain occurred in 30 pts, median NRS was 5,5 (range 4-9) and it was treated with opioids and tricyclic antidepressants. After PN diagnosis 18 pts required bortezomib/thalidomide dose reduction, 3 pts a temporary drug discontinuation and 5 pts interruption, while 39 pts continued their treatment with close neurological assessment. With a median follow up of 56,5 months (range 7-232) 28 pts showed PN resolution. In 6 cases PN worsened during bortezomib retreatment. *Conclusions.* Our study showed that clinical and electrophysiological examination are both useful in the early diagnosis of PN. Careful monitoring is required to guide the physician in dose adjusting, especially when bortezomib and thalidomide are used in combination, in order to avoid the development of grade 3-4 PN. Also the use of oral opioids and tricyclic antidepressants seems to be effective in reducing the intensity of the neuropathic pain.

PO-162

ABSOLUTE LYMPHOCYTE COUNT IS NOT A PREDICTOR FOR SURVIVAL IN NEWLY DIAGNOSED ELDERLY PATIENTS WITH MULTIPLE MYELOMA. FINAL RESULTS OF A MULTICENTER STUDY

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Background. Absolute lymphocyte count (ALC) > 1.400x10^9/L, as a surrogate marker of host immune status, has been reported to be an independent prognostic factor for clinical outcome in patients with previously untreated multiple myeloma (MM). However, most of the patients evaluated received stem cell transplantation (SCT); less evidence is available on elderly patients or those unable to SCT. *Aims.* To evaluate retrospectively the correlation between ALC, detected at the time of MM diagnosis, and clinical outcomes (first remission rate and overall survival) in patients older than 65 y.o. or those not eligible to SCT. Methods. Between 1998 and 2006, 166 consecutive patients were evaluated among four institutions; none of patients was neither uniformly treated nor part of a clinical trail. The primary endpoint was to assess the role of ALC, at the time of MM diagnosis, on overall survival (OS); secondary outcome was the correlation between ALC and rate of first complete remission. The OS was measured, by Kaplan and Meier analysis, from MM diagnosis to death or last follow-up. Patients that were lost to follow-up were censored in the survival analysis. Differences between survival curves were tested for statistical significance using the two-tailed log-rank test.

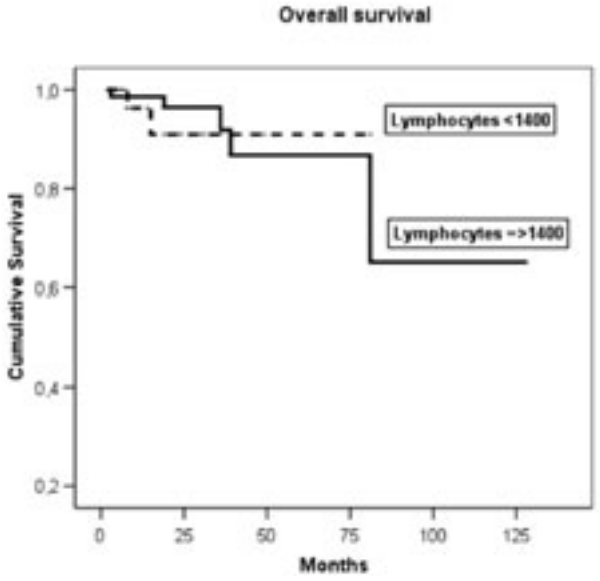

Figure 1. Overall survival among MM patients accordingly to baseline ALC at diagnosis.

Results. The median age for this cohort of 166 MM patients was 69.4 years (+ 10.8 years). Baseline characteristics (ISS, LDH, M Component, Haemoglobin Levels and Calcium) where not statistical significant. Three patients were lost to follow-up. Most of patients were treated with regimens containing Melphalan, Prednisone with/without Thalidomide. The median follow-up was 24.3 months (range: 1–81 months). At the

time of the analysis 71 patients had died. Of the 166 patients, 55 (33.1%) of the patients died of MM. ALC, as a continuous variable, was not identified as prognostic factor for overall survival (OS) (ALC < 1.400x10^9/L vs ALC > 1.400x10^9/L = HR 1.214 (95%CI: 0.222-6.650; P=0.823]). OS of MM patients with ALC > 1.400x10^9/L was 82 months vs 76 of those with ALC < 1.400x10^9/L (P= 0.23) (Figure 1). ALC did not influence response rate of first complete remission (ALC < 1.400 x10^9/L vs ALC > 1.400x10^9/L = HR 1.538 [95%CI: 0.662-3.570; P= 0.317]). Conclusions. This study showed that, in newly diagnosed MM, ALC is not an independent prognostic factor for OS and does not influence rate of first complete remission.

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HIGH CNA LEVEL AND OVER-EXPRESSION OF GENES INVOLVED IN RESPONSE MECHANISMS TO GENOTOXIC STRESS CHARACTERIZE NEWLY DIAGNOSED MULTIPLE MYELOMA (MM) PATIENTS CARRYING AMPLIFIED MDM4 AND/OR DELETED TP53

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Background. The p53 tumor suppressor pathway is tightly kept in check, or completely silenced in cancer cells. A potent inhibitor of p53 is MDM4, which is critical for control of p53 activity during response to stress and is often amplified in several types of tumors. Both TP53 mutations and del(17p) are infrequently detected in newly diagnosed MM; nevertheless they have been strongly related to patients (pts) survival. Recently, the adverse prognostic impact of chr.1q amplification has been reported in MM. *Aim*. To investigate the prognostic and biological role of del(17p) and/or amp(1q) in newly diagnosed MM pts treated with bortezomib-thalidomide-dexamethasone (VTD) as induction therapy prior to, and consolidation after, double autologous stem-cell transplantation (ASCT). *Methods.* 88 pts treated with VTD were analyzed by means of GEP (Affymetrix U133 Plus2.0 array) and unpaired analysis of copy number alterations (CNA) (Affymetrix 6.0 SNP array). *Results.* 30/88 pts (34%) carried a minimal amplification region of 1,1 Mb on chr.1q, which harbors MDM4. 9/88 pts (10,2%) carried a minimal deletion region of 482 Kb on chr.17, which harbors TP53. Pts were stratified into two subgroups according to the presence of amplified MDM4 and/or deleted TP53 (group A, 36 pts, or 41%) or the absence of both these abnormalities (group B, 52 pts, or 59%). Rates of best complete or near complete response were 81% and 63% in group A and B respectively, with time-to-best-response of 7.7 months (group A) vs. 12.7 months (group B) (P=0.04). Rates of relapse or progression were 58% and 27% for group A and B respectively (P=0.004), with a median progression-free survival of 39.6 months and not reached for pts of group A and B respectively (P=0.04). The average number of aberrations per group was higher in group A as compared with group B (191 vs. 117 CNAs, P=0.03). A comparison of expression profiles of the two groups of pts highlighted an overall deregulation of genes involved in response mechanisms to genotoxic stress, i.e damage sensor genes (ATM, RAD21), damage signal mediator genes (CHK1, MSH2, MSH5), genes involved in regulation of cell proliferation (CDKN2A, CDC14a) and anti-apoptotic genes (CASP6, BCL6, TP63) (one-way ANOVA, p <0,01). Finally, group A significantly over-express the transcription factor YY1, which is known to interact with p53, thus inhibiting its transcriptional activity. *Conclusions.* Pts carrying amplified MDM4 and/or deleted TP53 showed a significantly higher number of CNAs and the significant overexpression of genes involved in response mechanisms to genotoxic stress, as compared to pts lacking these chromosomal aberrations. This might account for the worse outcome of these group of pts. Amplification of MDM4 locus and over-expression of YY1 might contribute to maintain p53 in an OFF state by indirect mechanisms. Supported by: Ateneo RFO grants (M.C.) BolognAIL.

PO-164

NOVEL HSP-990 INHIBITORS REDUCE TUMOR BURDEN IN MULTIPLE MYELOMA MOUSE MODEL

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Multiple myeloma (MM) still remains an incurable disease, as CR may be achieved only transiently. Bone marrow microenvironment plays a key role by favouring MM cell survival. *in vitro* studies are not sufficient to test drug efficiency: animal models are important to develop new therapeutic strategies.We used 5TMM mouse model (Vanderkerken et al, Immunol Rev. 2003), which originates from spontaneously developed MM in elderly mice of C57BL/KalwRij strain, to test *in vitro* and *in vivo* two new Hsp-90 inhibitors (AUY-992 and Hsp-990) provided by Novartis Farma. These molecules had showed preliminarily a potent anti-cancer activity in *in vitro* models of leukemia, lymphoma and selected solid tumors, and synergistic effects with conventional cytotoxic drugs. *Methods.* To assess the anti-proliferative activity of Hsp-90 inhibitors on cells in vitro, we performed standard 2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt (XTT) colorimetric assay at different drug concentrations for 24 and 48 hours on different MM cell lines (5T33vtMM, RPMI-8226, KMS-11, U-266). We also studied apoptosis through Annexin-V/PI test and Caspase-3 cytofluorimetric detection method.We treated three month-old C57BL/KalwRij 5T33vtMM-bearing mice with different administration schedules. AUY-922 was tested at either 50 mg/kg i.p. once a week or 50 mg/kg i.p. twice a week; Hsp-990 was tested at either 10 mg/kg p.o. once a week or 10 mg/kg p.o. twice a week. All treatments started at day +10 from tumor cell injection. Mice were euthanized once developing paraplegia or any another sign of pain. Serum was collected at specific time points to test levels of mouse M-component IgG2b and VEGF by ELISA tests. *Results. in vitro* studies. Both AUY-992 and Hsp-990 had cytotoxic effect on all MM cell lines, with different IC50 values. Significant apoptosis was induced with low doses of AUY-992 (12.5 nM) in all cell lines in absence of dose-effect curve. Hsp-990 led to significant apoptosis at 12.5 nM dose only in U-266 and 5T33vtMM, but not in KMS-11 and RPMI-8226, which required higher concentrations of drug. Hsp-990 pro-apoptotic effect was dose-dependent. Thus, AUY-992 and Hsp-990 seem to act differently. *In vivo* studies. At day +45 after cell injection, both molecules and treatment schedules reduced significantly tumor burden, as measured by serum M-component and VEGF levels. We did not find any advantage in using higher doses of both drugs, rather signs of toxicity (necrosis in the side injection) in mice treated with AUY-992 twice/week. Hsp-990 was administered p.o., with no toxicity related to dose increase. However, the treatments did not modify OS. *Conclusion.*The reduction in tumor burden observed with AUY-992 and Hsp-990 used as single agents is a promising result, regarless the lack of OS improvement. The synergy of AUY-992 and Hsp-990 with other drugs will be further investigated. *Acknowledgments.* Thanks to K. Vanderkerken, who kindly provided 5T33vtMM cell line.

Myeloproliferative Syndromes

PO-165

MIRNA AND GENE EXPRESSION PROFILES OF JAK2V617F KNOCK-IN (KI) MICE REVEAL NEW GENE NETWORKS POTENTIALLY ASSOCIATED WITH MYELOPROLIFERATIVE NEOPLASMS (MPN)

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Introduction. To shed light on regulatory role of miRNAs in MPN, we performed a preliminary analysis of miRNA and gene expression profile in a KI inducible JAK2V617F mouse model. *Methods.* miRNA or gene expression profilings were concurrently performed, using the Exiqon miRNA array or Affymetrix Mouse Genome 430 2.0 Array, respectively, in TER119+ and GR1+ cells purified from JAK2V617F KI and JAK2wt mice. Potential miRNA targets were predicted in silico according to Targetscan 6.1. Then, the combined analysis of miRNA and gene expression profiles and target prediction was used to identify the subset of predicted taget genes most supported by expression data, namely by miRNA and target gene expression profiles anti-correlation. *Results.* We selected as differentially expressed those miRNAs with |log Fold Change(KI/WT)|>2: 20 miRNAs were modulated in GR1+ cells (10 up-regulated and 10 down-regulated) and 23 in TER119+ (11 up-regulated and 12 down-regulated). Among them, nine miRNAs were shared by both erythroid and myeloid cells: five were up-regulated (miR-27b, miR-106a, miR-26a, miR-301a, miR-16) and four down-regulated (miR-350, miR-33, miR-150, miR-let-7f). While some of them were already described as deregulated in MPN (Bruchova et al. first found a significant down-regulation of miR150 in PV; Guglielmelli et al. found increased levels of miR-16 in purified CD34+cells and erythroid cells generated *in vitro* from PV patients), none of the others have been previously implicated in MPN. We carried out the combined analysis on these nine miRNAs and their predicted targets. We found 125 targets whose expression was inversely correlated with that of the putative regulatory miRNA . Overall these include genes involved in pathways having functional relevance for MPN such as cell cycle, proliferation, apoptosis (60%) and epigenetic (20%) regulation. The 20% remaining are genes poorly characterized. Interestingly, we found that EZH2 and PIM-1 (both targets of the up-regulated miR-26a) were less expressed in JAK2V617F KI mouse compared to JAK2WT. EZH2 down-regulation is in agreement with the recent discovery of inactivating mutations and chromosome loss of the gene in MPN. Down-regulation of PIM-1, a known STAT-regulated gene that also functions as negative regulator of the JAK/STAT pathway, could result in disruption of this negative feedback loop further contributing to enhanced activation of JAK/STAT pathway. *Conclusions.* In summary, our study suggests that expression of the JAK2V617F mutation leads to miRNAs deregulation in KI mice and points to novel molecular mechanisms and candidate gene targets that may have relevance for the development of MPN. Further studies are in progress to understand the relation between miRNAs, targeted coding mRNAs and the JAK2V617F mutation in this mouse model as well as in primary human cells.

PO-166

LOW LEVELS OF T REGULATORY CELLS IN THE PERIPHERAL BLOOD OF PATIENTS WITH PRIMARY MYELOFIBROSIS ARE ASSOCIATED WITH SEVERE ANEMIA

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Introduction. Primary myelofibrosis (PMF) is a clonal, neoplastic disorder of the hematopoietic stem cells, characterized by bone marrow fibrosis, anemia, increased number of circulating CD34+ cells and splenomegaly. A subset of patients displays immune-related abnormalities that suggest an (auto)immune pathogenesis; in addition, a significant association between personal history of a broad spectrum of autoimmune disorders and subsequent risk of myeloproliferative neoplasm has been reported. T regulatory cells (Tregs) are identified as CD4+CD25+CD127low/neg cells. They define an immunosuppressive T-cell subset that prevents autoimmunity, regulates T-cell homeostasis, and modulates immune responses against a variety of pathogens. The involvement of Tregs in blood diseases has been studied extensively in acute leukemia and in the setting of hematopoietic stem cell transplantation and there is accumulating evidence that these cells also play a role in the regulation of hematopoiesis. Based on these premises, we decided to investigate the number of circulating Tregs in patients with PMF, and to correlate them with the main parameters of the disease. Patients with polycythemia vera (PV) or essential thrombocythemia (ET), and healthy subjects (HS) were used as controls. *Methods.* We tested 72 patients with PMF (17 were receiving either cytoreductive or steroid therapy), 5 with PV or ET, and 16 HS. All the subjects included in the study signed an informed consent. Tregs were evaluated by cytofluorimetric analysis of peripheral blood mononuclear cells. *Results.* The percentage of CD4+CD25+CD127low/neg cells was significantly lower (P=0.02) in patients with PMF (1.8%, range 0.1-10.9) than in HS (3.8%, range 0.8-11.5). In patients with PV or ET the percentage of CD4+CD25+CD127low/neg cells was comparable to that of HS (not shown). No significant correlation was found between the percentage of Tregs and therapy, disease duration, severity score, WBCs, PLTs, Hb, and IWG-prognostic score. However, among patients not receiving therapy (n=55), those with severe anemia (Hb < 10 g/dL) (n=8) had a percentage of Treg cells significantly lower than those with Hb levels equal or higher than 10 g/dl (n=47) (1.0%, range 0.4-3.6 vs 2.2%, range 0.1-10.9, respectively; P= 0.035), and those of HS (3.8%, range 0.8-5.3; P= 0.003). *Conclusions.* These preliminary data indicate a correlation between a low percentage of CD4+CD25+CD127low/neg Tregs and anemia in patients with PMF. Together with the well known improvement of anemia following immuno-suppressive therapy reported in some patients, they clearly point to a potential role of (auto)immunity as a cause of anemia in a subgroup of patients with PMF.

PO-167

ANGIOGENESIS IN PHILADELPHIA-NEGATIVE CHRONIC MYELOPROLIFERATIVE NEOPLASMS: IMMUNOHISTOCHEMICAL ANALISIS.

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Background. Increased bone marrow angiogenesis evaluated as immunohistochemical expression of angiogenic factors in bone marrow biopsy has been demonstrated in a variety of haematological disorders including Polycythemia Vera (PV), Essential Trombocythemia (ET) and Primary Myelofibrosis (PMF). In literature Myeloproliferative neoplasms angiogenesis is usually assessed using the expression of the Vascular Endothelial Grow Factor (VEGF) and CD34. Aim of this study was to evaluate bone marrow angiogenesis through immunohistochemical analysis, including angiogenic factors such as VEGF-receptor1 (KDR) and Matrix Metalloproteinases (MMP9). *Method.* Bone marrow paraffin embedded biopsies from 30 patients with Ph negative myeloproliferative neoplasm were examined (10 PV, 10 ET, 10 PMF). Main clinical features of the patients were the following: 60% of cases were male with a median age of 61 years (range 28-82); median white blood cells (WBC) Haemoglobin and platelets levels were respectively 9.5 x106/L (±6.8), 14.4 g/dL (±3.4), 572x10^6/L (±355). Splenomegaly was observed in 9 patients (30%) particularly in PMF (7 of 10); JAK2 mutation was detected in 23 patients (69%). To identify BM microvessels, we use anti-CD34 staining, and the following antibodies were used to assess BM angiogenesis: Matrix metalloproteinase 9 (MMP- 9), VEGF, VEGF receptor (KDR/flk-1). Antibody expression was evaluated as percentage and absolute number of positive cells in a total of eight consecutive areas at 400x magnification. To avoid any bias related to variations in BM cellularity, we normalized the expression calculating the "antibody expression index" (% BM cellularity x % Ab positive cells/104). *Results.* Median expression of MMP9 and VEGF was higher in PMF than in PV and ET. Statistical analysis showed a significant statistical difference of these two antibodies (respectively P=0.005 and P=0.002) but when antibody expression was normalized for the cellularity only MMP9 maintained a the statistic significance (P=0.01). Bone marrow biopsy specimens from

patients with MF showed an increased vascularity comparing to PV and TE cases. *Conclusion*. Our results suggests a higher grade of vascularisation in PMF. MMP9 and VEGF expression was higher in PMF, comparing to ET and PV, without a proportional increase of KDR. Further studies on a large cohort are necessary to correlate our preliminary data with clinical characteristics and clinical outcome of negative myeloproliferative neoplasms.

PO-168

A COMPLETE REMISSION INDUCED BY VALPROIC ACID IN A PATIENT WITH ESSENTIAL THROMBOCYTEMIA JAK2V617F-POSITIVE

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Introduction. Polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) are myeloproliferative neoplasms characterized by excessive proliferation of myeloid/erythroid lineage cells and JAK2V617F somatic mutation. Since current treatments of MPNs are unlikely to cure or offer remission to patients, there is a clear necessity of new therapies. Histone Deacetylase inhibitors (HDACi) are a new class of drugs with a potential activity in this group of disease. Among these, valproic acid (VPA) is a well tolerated and long since used drug for the treatment of epilepsy and bipolar disorders. At concentration equivalent to those used by neuro-psychiatrists (> 50 g/mL), VPA acts as a powerful HDACi, that has been shown to be promising in the treatment of both solid and hematological tumors. Therefore herein we present a 52 years-old woman with JAK2V617F ET who received VPA for a bipolar disorder. *Methods.* Diagnosis of ET was done 2 years before our observation, at the time of an episode of angina pectoris treated with four coronary by-passes. Patient started treatment with antiplatelet drugs and hydrossiurea (HU). However, due to a severe intolerance, HU was intermittently assumed never reaching therapeutic concentration and disease control. During this period, the patient developed heightened mood (euphoric and irritable), decreased need for sleep and hyperactivity. A psychiatric evaluation allowed to made the diagnosis of bipolar disorder type II. Thus the patient definitively stopped HU and started VPA at increasing doses, reaching a therapeutic serum level of 76 g/mL after four months with a dosage of 1500 mg/day and achieving the complete remission of her manic phase of the bipolar disorder.

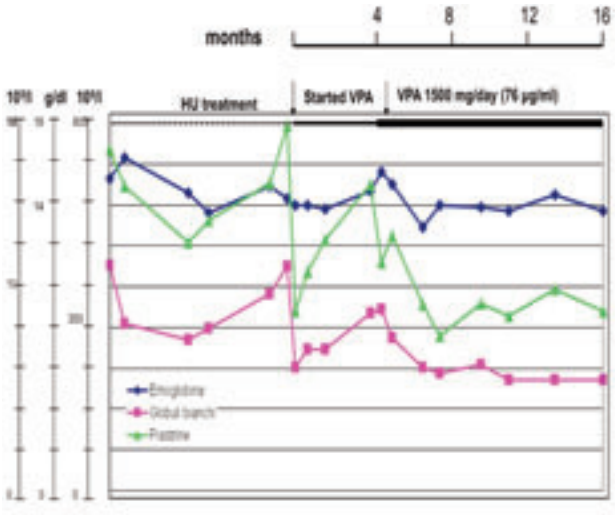

Figure 1. The clinical course.

Results. As for the clinical course of ET, her hemochromocytometric values before VPA were: Hb 14.3 gr/dL, WBC count 11.3x10^9/L and platelets 793x10^9/L. Two months after reaching the VPA therapeutic level we observed a significant decrease in the values of Hb, WBC and Platelet that resulted 12.9 gr/dL, 6.3x10^9/L and 403x10^9/L, respectively. Since, these values are remained stable after more than one-year of VPA treatment, the patient has been classified in complete remission (CR) of disease (figure 1). Molecular monitoring of disease during VPA treatment was performed by evaluating JAK2 gene dosage and JAK2WT and JAK2V617F transcript levels at diagnosis and after 4, 8 and 12 month from the start of VPA. Although, the JAK2 gene dosage remained constant upon treatment,, JAK2WT and JAK2V617F transcript levels significantly increased at each time points. *Conclusion.* Data herein reported demonstrated, for the first time, the achievement of CR induced by the HDACi VPA in a ET patient intolerant to HU. The findings that JAK2WT and JAK2V617F transcript levels increased, despite the achievement of therapeutic response, support that JAK2 is not fully implicated in the pathogenesis of Ph(-) MPN.

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INFLUENCE OF JAK2 V617F MUTATION STATUS AND ALLELE BURDEN ON SURVIVAL FREE OF THROMBOSIS AND MYELOFIBROSIS IN ESSENTIAL THROMBOCYTHEMIA

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Introduction. Fifty to sixty percent of patients with essential thrombocytemia (ET) harbor JAK2 V617F mutation. The impact of this mutation and its allele burden on patient clinical outcomes is still being debated. The aim of this study was to determine whether the presence of JAK2 V617F mutation and its allele burden correlate with major clinical outcomes in patients with ET. *Methods.* A single-center retrospective study was performed on patients with a diagnosis of ET made according to WHO criteria. The JAK2 V617F mutation was firstly investigated by qualitative allele-specific PCR on DNA extracted from granulocytes; moreover in positive cases the allele burden was quantitatively evaluated by means of JAK2 Mutaquant Kit (Ipsogen).

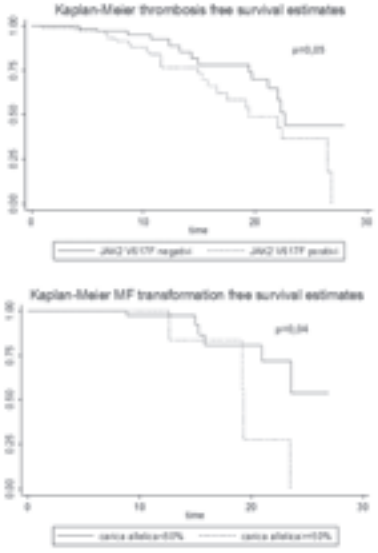

Figure 1. The clinical course.

Results. The population was composed of 184 patients: mean age at diagnosis was 51,3 years (range 14-84 years), female/male ratio was 1,71, and mean follow-up from diagnosis was 9,5 years (range 0.5-28). In the 103 V617F positive patients (56% of total cohort), an allele burden greater than 50% was found in 13 patients (12,6%). Thrombotic events were registered in 39 patients (21.2%) and evolution to myelofibrosis (MF) occurred in 17 patients (9.2%) during the course of their disease. A shorter thrombosis free survival time was associated with JAK2 mutation status (P=0,05), whereas myelofibrosis free survival time was correlated to patient JAK2 allelic burden >50% (P=0,04). *Conclusions.* The study showed that in patients affected by ET an analysis of the allele burden is required to assess the risk of MF transformation, while it is not relevant to determine a higher thrombotic risk, which is only related to the presence of JAK2 V617F mutation.

PO-170

PERIPHERAL BLOOD CD34+ CELLS IN IDIOPATHIC MYELOFIBROSIS

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Background. Idiopathic Myelofibrosis (IMF) is chronic myeloproliferative neoplasm characterized by constitutive mobilization of hematopoietic stem cells (HSC) and progenitor cells (HPC) into the peripheral blood (PB). The interaction between the chemokine CXCL12 and its receptor CXCR4 plays a pivotal role in determining the trafficking of CD34+ cells between the bone marrow (BM) and the PB. IMF is associated with downregulation of CXCR4 by CD34+ cells due to epigenetic events. Altered gene expression was corroborated by the detection of abnormally high CD9 or CD164, and low CXCR4, membrane protein expression in IMF CD34+ cells. Moreover, endothelial precursor cells (CD34+/CD133+) are increased in the blood of a subset of patients with IMF, and peripheral endothelial cells bear the same molecular markers as hematopoietic cells, suggesting a primary role of pathological endothelial cells in this disease. *Methods.* We evaluated, by flow cytometry, the number of CD34 positive cells in peripheral blood and the expression of CXCR4, CD9, CD117 and CD133 on these cells. In our institution we are following 22 patients affected by IMF, according to WHO criteria (M: 14, F: 8; median age: 55 years, range: 48-66 years). *Results.* In all patients, at diagnosis, we found a high count of CD34+ cells in PB (greater than $15x10^6$/L; median: $2,4x10^6$/L, range: $1,8\text{-}3,2x10^6$/L) compared with normal controls and other Philadelphia-negative chronic myeloproliferative neoplasms. In all cases CD34+ cells were negative for CXCR4 while expressing high intensity CD9. About 40% of CD34+ cells expressed CD133, while 20% expressed CD117 at low intensity. In no case was detected coexpression of CD133 and CD117, suggesting a simultaneous presence of two distinct hematopoietic progenitors, endothelial progenitors and myeloid progenitors. We monitored every 6 months the phenotypic pattern of CD34+ cells, and after 36 months have seen an increase of myeloid precursors (CD34+/CD117+: 45,7%) compared with a reduction of endothelial precursors (CD34+/CD133+: 15,3%). *Conclusions.* By comparing these findings with other clinical data, our results seem to confirm that, according to the natural history of disease from an initial stage towards a fibrotic phase (pancytopenia and/or splenomegaly), there was a change in PB CD34+ cells. Immunophenotypic profile of PB CD34+ cells is associated in IMF with patients' clinical characteristics and may have potential prognostic application.

PO-171

DEVELOPMENT OF LYMPHOPROLIFERATIVE DISORDERS IN PATIENTS WITH A DIAGNOSIS OF MYELOPROLIFERATIVE NEOPLASMS: THE EXPERIENCE OF THE DIVISION OF HEMATOLOGY OF THE SAPIENZA UNIVERSITY OF ROME

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Introduction. It is commonly believed that the incidence of secondary tumors, including those of the hematopoietic system, is increased in patients affected by myeloproliferative neoplasms (MPN). In particular, the association of MPN with a disorder of the lymphoid system has been sporadically reported in the literature. Aim of this analysis was to assess the association of Philadelphia chromosome-negative (Ph-) MPN with lymphoproliferative disorders (LD) detected at our Center. *Patients and Methods.* We retrospectively analyzed 1000 patients affected by Ph-MPN, 350 with polycythemia vera (PV), 450 with essential thrombocythemia (ET) and 200 with primary myelofibrosis (PMF) referred to the Division of Hematology of the "Sapienza" University of Rome in the period 1980 to 2011. Information on patient characteristics (gender, date of birth, age at diagnosis), baseline clinical characteristics, and JAK2 V617F mutational status were retrieved from the medical records and computerized, together with information on newly diagnosed LD. The diagnosis of LD was carried out according to the WHO criteria and, in all cases, the original diagnosis was critically reviewed using chart records, laboratory records as routine tests and immunophenotyping, bone marrow and/or lymph node biopsies when recommended. *Results.* We identified 40 patients with MPN (52.5 % women and 47.5% males, median age 62 years) who developed a LD over their lifetime; among them, 17 had a diagnosis of ET, 12 of MFI and 11 of PV. The JAK2 V617F mutation was found in 12 of the 25 cases analyzed at MPN diagnosis. No family history of hematologic malignancies was reported in any of these patients. Of the 40 LD evaluated, 29 monoclonal gammopathies (M-GUS), 4 chronic lymphocytic leukemias (CLL), 3 non-Hodgkin lymphomas (LNH), 2 monoclonal B-cell lymphocytosis (MBL), 1 T cell large granular lymphocyte (T-LGL) LD and 1 multiple myeloma (MM) were reported. The median interval time from diagnosis of MPN to LD was 55.9 months (range, 0-338), whereas 26 of the 40 patients (65%) had been on cytoreductive treatment when the LD was diagnosed for a mean time of 83 months. *Conclusions.* Although the association between Ph-MPN and LD is a relatively uncommon event, the results here presented support the theory of the global genetic instability of the pluripotent hematopoietic progenitor in the MPN context. This confirms the importance of performing, in patients affected by hematological disorders at diagnosis, an integrated diagnostic work-up using different laboratory techniques, with the aim of extensively characterizing the biological properties of the disease with obvious prognostic and therapeutic implications.

PO-172

RAPID, HIGH SENSITIVE JAK2V617F MUTATION DETECTION IN CHRONIC MYELOPROLIFERATIVE NEOPLASMS BY ISOTHERMAL FLUORESCENT AS-LAMP

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Introduction. The detection of JAK2V617F mutation is mandatory in Chronic Myeloproliferative Neoplasms (MPNs) diagnostic work-up and therefore the demand of molecular techniques for this purpose is constantly increasing. We have developed a novel isothermal method based on the LAMP technology that improves the reliability, rapidity and sensitivity of JAK2V617F detection. *Methods.* The AS-LAMP assay consists in a fluorescent non-PCR reaction able to selectively amplify low amount of JAK2V617F mutated DNA in a single step. Simultaneously, the endogenous Abelson gene (ABL) can be amplified as internal control. JAK2V617F and ABL products, characterized by different melting temperatures, can be distinguished by a melting analysis. *Results.* The duplex JAK2V617F/ABL AS-LAMP demonstrated 100% specificity on 315 DNA replicates from cell lines proved wild-type for JAK2V617F. The assay sensitivity, established on serial dilution of mutant JAK2V617F DNA into wild type DNA (from UKE-1 and B-JAB cell lines respectively), reaches 0.05%. The JAK2V617F/ABL AS-LAMP was validated on DNA extracted from granulocytes of patients affected by PV (n=6), ET (n=7) and other hematologic malignancies (n=7) demonstrating 100% concordance with conventional Allele Specific Oligonucleotide PCR (ASO-PCR). Moreover, the high robustness and sensitivity of the method allows the correct detection of 17 DNA positive samples extracted directly from whole blood, with no need of further granulocytes enrichment. All the negative controls (ET=5, MF-Post-ET=3, healthy donors=10) resulted negative for JAK2V617F mutation in presence of an optimal ABL gene amplification. Finally, a direct relationship between the amount of mutant target tested and the height of peaks obtained after melting analysis has been consistently observed. *Conclusions.* The implementation of the AS-LAMP assay to a fluorescent, duplex format produced a specific, sensitive, more rapid and reliable real-time assay for the JAK2V617F detection. Moreover, the direct relationship between the amount of target and the height of the melting peaks can lead to future improvement of the assay to a quantitative format. AS-LAMP represents a powerful tool for overcoming the limitations of the conventional PCR techniques actually employed in the routine

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ALL-TRANS RETINOIC ACID (ATRA) SYNERGIZES WITH INTERFERON-ALPHA (IFN) IN CHRONIC MYELOPROLIFERATIVE NEOPLASMS (MPN)

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Introduction. Although IFN has proven efficacy in Polycythemia Vera (PV) and Essential Thrombocythemia (ET), many patients experience unacceptable side effects. ATRA synergizes with the inhibitory effect of

IFN on the growth of malignant and normal hematopoietic cells. Their pathways converge on the promoter of STAT1 that induces IRF-1 transcription. This tumor suppressor exerts pro-apoptotic effect trough TRAIL and caspase activation. ATRA activates TRAIL expression also by IRF1 independent mechanisms. These drugs cooperate in blocking cell cycle by inducing p21WAF1/CIP1. This study aimed to *in vitro* investigate the synergistic activity of ATRA and IFN in MPN, in order to reduce IFN doses and its related side effects. *Methods.* HEL and SET2 cell lines characterized by JAK2V617F mutation were treated with a scalar dose of IFN (1-10000U/mL), ATRA (0,1nM-1x106nM) and a combination of the two drugs. Proliferation rate, apoptosis and cell cycle were evaluated after 24-48-72-96-120-168 hrs. CD34+ cells purified from BM of four MPN patients (PV=1, ET=3) at the time of diagnosis were plated (2x103cells/mL) in semi-solid methylcellulose medium and were incubated with IFN (10000U/mL), ATRA (0,01 mM) or their association. Colony forming cell assay (CFC) was performed at day 14. RAR , , and RXR , , gene expression was evaluated both for cell lines and CD34+ cells from MPN patients. Results: We observed in SET2 cell line an antiproliferative effect of IFN (10000U/mL) and ATRA (0,01 mM) after 120-168 hrs of treatment. Apoptosis assay showed that, at this concentration, HEL cell line is less sensitive to IFN (P=0,0002), ATRA (P=0,0026) and their combination (P=0,00001) than SET2. RT-qPCR showed a significant down- regulation of RAR and up-regulation of RAR in HEL respect to SET2. This differential expression of RAR subunit may explain the different responsiveness to ATRA. IFN and ATRA treatments were effective in inducing SET2 growth inhibition (P=0,0006; P=0,0008) and apoptosis (P=0,001; P=0,04) compared to untreated cell line. Moreover, their combination synergistically increased both growth suppression (IFN vs ATRA+IFN P=0,0001 and ATRA vs ATRA+IFN P=0,0006) and apoptosis (IFN vs ATRA+IFN P=0,036 and ATRA vs ATRA+IFN P=0,00001). Cell cycle analysis revealed a reduction of SET2 cells in S phase after IFN (6,2%) and IFN+ATRA treatment (3,9%), compared to untreated cell line (13,2%). CFC showed that IFN reduced CFU-GM (54+34; P=0,04) without affecting BFU-E and CFU-GEMM formation, while ATRA alone inhibited BFU-E (34+10 P=0,01) growth. Although the addition of ATRA seems to neutralize the IFN inhibitory effect on CFU-GM (127+24 P=0,25), their combination suppresses BFU-E (3+2 P=0,003) and CFU-GEMM (23+7 P=0,004). Conclusion: These results indicate that, the *in vitro* combination of IFN and ATRA acts synergistically in inhibiting SET2 cell line and in suppressing BFU-E and CFU-GEMM growth and suggests that it has a potential interest for the treatment of PV and ET.

PO-174

INTERFERON ALPHA EFFECTIVENESS IN ESSENTIAL THROMBOCYTHEMIA

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Background. Interferon-alpha (IFN) has shown significant activity in MPN. In ET it is able to rapidly normalize platelet and leukocyte counts, as well as organomegaly. IFN is the only therapy known to induce complete molecular remission with the reduction or the suppression of tumor burden. Despite its effectiveness, many patients discontinue IFN therapy due its side effects. Their incidence and severity are dose-related and the most common are flu like syndrome, myalgia, headache, fatigue, myelosuppression, liver abnormalities, autoimmune disease and depression. IFN has no leukemogenic effects and is safe in pregnancy. We hereby report our monocentric experience. *Methods.* From 1992 until today, the Division of Hematology of the Federico II University Medical School, Naples, Italy, followed 365 ET patients (diagnosis according to PVSG criteria and subsequently with 2008 WHO criteria); 257 patients were treated with cytoreduction: 64.5% with hydroxyurea, 23.5 % with IFN, 10% with anagrelide and 2% with busulfan. Patients treated with IFN received subcutaneous injection at induction dose of $3x10^6$ units 5 times a week, reduced on the basis of clinical-hematological response. *Results.* After a median follow up of 36 months, of the 60 patients receiving IFN, 60% achieved complete hematological response (CHR) according to the European Leukemia Net criteria; of these 13% were taken off therapy and 39% are still receiving very low doses of IFN ($3x10^6$ units every 7-15 days). As many as 20% experienced partial hematological response (PHR). The median time to response was 2.6 months. Response was not influenced by age (P=0.06), gender (P=0.4), baseline values of hematocrit and hemoglobin normalized for the gender, platelet count (P=0.29), WBC count (P=0.1). Furthermore JAK2V617F mutational status does not influence the response. Under IFN therapy no patients had thromboembolic or hemorrhagic event, independently of their cardiovascular risk. As many as 22 patients experienced flu-like symptoms, 3 dermatitis, 3 alopecia, 7 autoimmune thyroiditis; of them only 2 patients had hypothyroidism and had to suspend IFN treatment. Average spleen volume at the baseline was 495 ml; after a median follow-up of 18 months it was 472 ml, with no differences in volume enlargement in CHR/PHR compared to non responsive patients. Bone marrow fibrosis grade (according to Thiele) did not worsen after IFN treatment in either patients who achieved CHR or PHR. We are unable to evaluate data about non responsive patients, because this parameter could be influenced by their second line treatment. Conclusion: IFN was safe and effective for patients with ET. The ability of IFN therapy to control and modify the clinical course of the disease is confirmed by our data. This response is sustained for prolonged periods in some patients, even after therapy discontinuation. IFN should be considered as election treatment even in young patients, for its lack of leukemogenic and teratogenic effects.

PO-175

LNK MUTATIONS IN JAK2 NEGATIVE ERYTHROCYTOSIS

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Introduction. Recent studies demonstrated the occurrence of LNK (SH2B3) mutations in MPN patients and in subjects with JAK2 mutations-negative erythrocytosis. In this study we analyzed LNK exons 2, 3 and 4 in 89 patients with JAK2 negative erythrocytosis to determine whether LNK mutations could associate with this clinical phenotype. *Methods.* We genotyped LNK exon 2 (by direct sequencing) and exons 3 and 4 (by High Resolution Melting Analysis and confirmation by direct sequencing), encoding the Pleckstrin Homology domain using granulocyte or whole blood DNA. Statistical analysis was performed with 2 test e T-student test using SPSS software. *Results.* This was a cohort of patients with erythrocytosis who tested negative for JAK2 V617F and exon 12 mutations and did not fulfill the WHO criteria for Polycythemia Vera. We detected 4 different mutations in 6 patients (6.7%). Three patients exhibited the previously described heterozygous 622G>C substitution in exon 2 leading to E208Q aminoacid change. Other two patients displayed mutations in exon 2: an heterozygous 464C>T substitution leading to P155L mutation and an heterozygous 639C>A change causing S213R aminoacid substitution. The latter alteration occurs at the same base position of SNP rs111360561 (S213S) that however does not determine an aminoacid change. The sixth patient harbored a 820A>G substitution in exon 3 leading to T274A mutation. We did not found mutations in exon 4. Although we could not test germline DNA none of the novel discovered mutations was listed in the NCBI dbSNP or in the 1000genomes Project variation database. We analyzed the clinical features of our study cohort to determine if the presence of LNK mutation could affect any characteristic. Our patients group had a median age at diagnosis of 57 (18-79), average WBC count of 7.55 10^9/L (±2.3), Hb value of 17.25 g/dL (±1.17), average Ht value of 51% (± 3.24), platelet count of 266 10^9/L (±116) and average EPO value of 12.58 mU/mL (± 6.56). LNK mutated patients seem to be older at the time of diagnosis in comparison with wt patients, although there is no statistical significance. Likewise we failed to identify any statistically significant difference in any other clinical parameter in LNK mutated patients when compared to non mutated patients, probably due to the small number of mutated cases. *Conclusions.* This study confirms and extends previous reports that LNK mutations occur at low frequency in JAK2 negative erythrocytosis. While their clinical significance still remains to be clarified, these mutations could represent an usefull diagnostic test to reveal an underlying clonal disease in patients negative for others more frequent mutations occurring in PV.

PO-176

COULD HYPOXIA INCREASE THE PREVALENCE OF THROMBOTIC COMPLICATIONS IN POLYCYTHEMIA VERA?

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Introduction. Thromboses represent a major cause of morbidity and mortality in Polycythemia Vera (PV) but the contributing mechanisms are not fully described. *Methods.* To evaluate whether environmental conditions such as altitude/hypoxia could impact thromboses history, we retrospectively analyzed thrombosis history in 71 PV patients living at an elevation of 1.500 meters or more in the Salt Lake City area (SLC) and 166 PV patients living near sea level in the Baltimore area (BLM). The SLC cohort was older with a longer disease duration. No significant differences in type of anticoagulation therapy or prothrombotic factors were present between the two cohorts. Results: After adjusting for age, sex and disease duration, SLC patients experienced an estimated 3.9-fold increase in the odds of a history of thromboses compared to BLM patients (95% confidence interval 1.8- 7.6; P=0.0004). A history of cardiovascular event was present in 58% of the SLC patients compared to 27% of the BLM patients (P<0.0001). Before diagnosis thromboses occurred in 18% and 4% of the SLC and BLM groups respectively (P= 0.003). No correlation between JAK2V617F allele burden and thrombosis was observed in this study. Conclusion: This retrospective study suggests that even moderate hypoxia associated with 1.500 meters elevation should be considered as independent prothrombotic risk factor. This observation needs to be confirmed by prospective studies.

PO-177

SPLENOMEGALY, OSTEOBLASTIC LESIONS AND UNEXPLAINED ANAPHYLACTOID SHOCK: A CASE REPORT

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Introduction. Systemic mastocytosis (SM) results from a abnormal clonal proliferation of mast cells (MCs) in more extra-cutaneous organs. Symptoms results from MC-derived mediators and from destructive infiltration of MCs. SM is a persistent disease in which a somatic c-kit mutation at codon 816 is usually detectable in MCs and their progenitors. A case with SM, c-kit positive, is discussed. *Methods.* A 45-years-old woman with mild anemia, hypotension, hot flush and splenomegaly (abdominal ultrasound showed spleen of 15x7x6 centimeters); physical examination indicated splenomegaly. Blood count was as follows only mild anemia (Hb 11.2 grams per deciliter, VCM 93 fentoliters); markers for cancer were negative and virologic tests were normal. CAT scan showed splenomegaly, 1.5 centimeters abdominal multiple lymphoadenopathy, 2 centimeters bilateral lynphonode axillary, multiple osteoblastic lesions and a solitary nodule is present in right lobe of thyroid. Skeletal radiogram revealed osteoblastic lesions and an small lytic lesion tibia. PET was positive in bilateral axillary, stomach and thyroid gland. Thyroid tests were normal and Abanti-TG and Abanti-TPO were absent. Peripheral smear showed anisopoikilocitosis and teardrop erytrocytes. Bone marrow aspirate-biopsie revealed the 50 per cent of disseminatted dense infiltrates of maste cells; blood marrow molecular test was positive for D816V mutation c-kit and negative for FIP1L1-PDGFRalfa while blood marrow immunophenothypic and cytogenetic test not showed alterations. Serum tryptase level test was upper. Gastroscopy and biopsy revealed helicobacter-positive gastritis and the thyroid biopsy was negative for malignant disease. *Results.* The patient was admitted to Onco-Ematologyc Center of Modena where she started the follow-up and the self-administration of epinephrine on demand has been recommended. *Conclusions.* The clinical course is variable. The therapy if necessary consists of antihistamines and mast cell membrane-stabilising compounds supplemented with medications targeted ad specific symptoms and complications. The use of "Kit-targeting"tyrosine has also been suggested but the D816V mutation c-kit is associated with relative resistance against STI571. Therefore, these patients require alternative targeted drugs or new drug-combinations. In case of anaphylactoide episodes, the self-administration of epinephrine on demand has been recommended as an appropriate approach.

PO-178

ANAGRELIDE ALONE REDUCES THE THROMBOTIC RISK IN PATIENTS WITH ESSENTIAL THROMBOCYTHAEMIA (ET)

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Background. Essential thrombocythaemia (ET), the most often occurring myeloproliferative disorder is a clonal malignant disorder arising from stem cell. The course of the disease is complicated by some severe thrombotic events and far less commonly by haemorrhagic phenomena. Treatment of ET consist of antiplatelet drugs (e.g. aspirin) and lowering platelet count (hydroxyurea or interferon alpha). Anagrelide is an oral imidazoquinazoline agent which is indicated in Europe for the reduction of elevated platelet counts in at-risk patients with essential thrombocythaemia who are intolerant of or refractory to their current therapy. *Methods.* In our institution we are following 120 patients affected by ET (71 females and 49 males; median age 48 years, range: 27-82) according to PVSG criteria and WHO classification. 67 out of 120 patients were classified as low-risk, 28 as intermediate-risk and 25 as high-risk. The Val617Phe point mutation of Janus Kinase 2 gene (JAK2V617F) was found in 69 patients. *Results.* Anagrelide was used in 25 patients with ET from Jan. 2010 to April 2012. Anagrelide, in the average dose of 2,0 mg (range: 1,0-3,5 mg), reduced platelet count in all patients. Median time of response was 3-4 weeks. Complete remission (platelet count < or = $450x10^9$/L) was achieved in 22/25 patients, and only three patients had platelet count slightly above $450x10^9$/L (but less than $600x10^9$/L). During the first two months of treatment with anagrelide some mild and transient side effects were noticed, e.g. headache in 7 (28%), fluid retention in 6 (24%), palpitations in 5 (20%), and diarrhoea in 2 (8%) patients, but all of them continued therapy. During anagrelide treatment, patients did not receive aspirin in order to avoid possible bleeding complications. We have found no case in thrombotic events. *Conclusions.* Anagrelide proved to be a safe and effective drug for pretreated ET patients. Moreover, anagrelide reduces significantly the risk of thrombosis without concomitant administration of aspirin. The positive results described in several studies may well lead to the next use of anagrelide as first line treatment in patients with high risk ET.

Cytogenetics and Molecular Analysis

PO-179

REVERSE PHASE PROTEIN MICROARRAYS FOR BIOMARKER AND THERAPEUTIC TARGETS DISCOVERY IN PEDIATRIC BCP-ALL: FOCUS ON THE AMPK PATHWAY

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Introduction. The outcome for children with ALL is improved in the last few decades, but 30% of patients still resist to conventional therapies. For many malignancies the signal transduction pathways (STPs) involved in the neoplastic process are not yet identified. Our study aims to provide such information in pediatric BCP-ALL patients using phosphoproteomics analysis. After this screening we focused our attention in the evaluation of AMPK as a new druggable target. *Methods.* Reverse Phase Protein Microarrays (RPMA) were employed to profile the activation status of 92 proteins in 118 pediatric BCP-ALL patients at diagnosis. Patients subgroups were compared to find differentially activated STPs. AMPK was inhibited using the commercial inhibitor Compound C in 4 BCP-ALL cell lines: SEM and RS4;11 carrying the MLL-AF4 translocation, MHH-CALL-2 and MHH-CALL-4 without recurrent chromosomal aberrancies. Cell proliferation was assessed by MTT assay and apoptosis was determined by flow cytometric analysis. Apoptosis features were analyzed by WB, immunofluorescence and flow cytometry. AMPK 1 was specifically silenced by lentiviral transfection of shRNA plasmids. In addition, 8 primary samples were treated with Compound C and apoptosis was measured. The effects of the combination of Compound C and drugs used in ALL treatment were evaluated in SEM cells. *Results.* We observed in poor clinical prognosis patients an increased activation/expression of several proteins involved in cell proliferation and survival. Our main finding concerned children with MLL (Mixed Lineage Leukemia) rearrangements. We identified a MLL-specific hyper-activated pathway that through AMPK phosphorylation leads to the activation of BCL-2. We thus investigated the role of activated AMPK in supporting MLL-rearranged leukemia cell survival. AMPK inhibition resulted in massive apoptosis selectively in MLL-rearranged cell lines. We silenced AMPK 1 through two different shRNAs and, as expected, we observed that silenced MLL-rearranged cells undergo more apoptosis than non- translocated cells. Compound C-induced apoptosis associated with mitochondrial membrane depolarization, Cytochrome c release and Caspase-9, -3 and -7 cleavage, indicating intrinsic apoptosis pathway activation. MLL-rearranged primary cultures responded very well to AMPK inhibition undergoing much more apoptosis than non-translocated patients. Moreover, AMPK inhibition resulted to synergistically enhance the antiproliferative effects of vincristine, daunorubicine and cytarabine. *Conclusions.* This study emphasizes the importance of protein pathway activation mapping analysis of clinical specimens as a route for discovery of functional derangements. With our research we also provide new insights into the role of AMPK in cancer. AMPK can be considered as a new drug target in MLL-rearranged leukemias and specific kinase inhibitors should be further studied in order to make new therapeutic options available for these poor prognosis patients.

PO-180

EVI1 REARRANGEMENTS IN A SUBSET OF PATIENTS WITH 3Q CHROMOSOMAL ABNORMALITIES WITHOUT EVIDENT 3Q26 CYTOGENETIC INVOLVEMENT

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Introduction. Overexpression of EVI1 has been associated with a poor prognosis in myeloid malignancies. The main mechanism for EVI1 overexpression is the rearrangement of the chromosome region 3q26, where EVI1 is mapped. Moreover EVI1 overexpression has been associated with distinct cytogenetic subgroups, such as normal karyotype and 7/7q-, in absence of observable abnormalities of 3q26 band. In some cases FISH analysis has evidenced cryptic EVI1 rearrangement. The association of EVI1 expression and 3q abnormalities other than 3q26 remains to be elucidated. The aim of this study is to evaluate the association of EVI1 expression and 3q abnormalities other than 3q26. *Methods.* The cases were selected according to the presence of 3q abnormalities or -7/7q- by conventional cytogenetics (CC). Subsequently patients were analyzed by FISH using EVI1 Breakapart probes (Cytocell, UK), and by relative quantitative RT-PCR (qRT-PCR). *Results.* By FISH we analyzed 63 patients showing 3q abnormalities and 30 patients showing -7/7q-. Eighteen cases showed classical inv(3)/t(3;3), 11 balanced 3q26 translocations, 34 balanced and unbalanced 3q abnormalities involving different loci from 3q26. EVI1 rearrangements were detected in 16 patients with inv(3)/t(3;3), 8 with balanced t(3q26) and 8 with other alterations of 3q. No EVI1 rearrangement was detected in the group with -7/7q-. In detail, among the patients with t(3q26) 4 demonstrated a t(3;21)(q26;q11), 1 a t(2;3)(p21;q26), 1 an inv(3)(p13;q26), 1 showed a t(3;7)(q26;q22), and the last one had a thus far not known t(1;3)(q32;q26). Among the patients with others 3q rearrangements: 2 revealed a t(3;21)(q21;q26), 1 showed a cryptic t(3;6)(q26;q25), 1 a t(3;8)(q21;q22) and other 4 patients had 4 new translocation involving EVI1 respectively: t(3;14)(q21;q24), t(3;14;16)(q21;q24), t(1;3;13)(p35;q?;q?) and a cryptic t(3;11)(q26;q23) in a unique ALL case with del(3)(q26q28) by CC. Further metaphase FISH analyses revealed a two-step or complex mechanism in the generation of EVI1 rearrangements that results in absence of 3q26 region involvement by CC. Moreover, EVI1 expression was measured by relative qRT-PCR in 18/32 cases with material available. In all investigated cases except for the ALL case with t(3;11) EVI1 expression was elevated (median=23.3) (range 2.6-226.5) compared to median EVI1 expression in 10 normal bone marrow [median=0.08 (range 0.03–0.86) (values are expressed as 2- Ct)]. Discussion. EVI1 expression and FISH analysis identify a group of patients with cryptic or complex EVI1 rearrangements, which apparently did not involve the region 3q26 by chromosome banding analysis. These observations indicate the importance of screening for EVI1 rearrangements and these method are particularly appropriate in hematological malignancies with chromosome 3q abnormalities even in absence of 3q26 involvement. Supported by University of Bologna RFO 2010-2011, Fondazione del Monte di Bologna e Ravenna and Bologna AIL.

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MULTIPARAMETRIC FLOW CYTOMETRY FOR DETECTION OF MINIMAL RESIDUAL DISEASE IN ACUTE MYELOID LEUKEMIA

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Introduction. Multiparametric flow-cytometry (MFC) may be used to assess the level of minimal residual disease (MRD) as a potential prognostic factor in acute myeloid leukemia (AML) patients in morphological complete remission (CR). *Patients and Methods.:* Forty-two consecutive, unselected, non-M3 AML patients, diagnosed at the Department of Haematology, Spedali Civili di Brescia between June 2010 and April 2012, and treated according to NILG protocol (AML BG00) or GIMEMA protocol (AML17), underwent MRD quantification by six-color MFC. The analysis was performed on fresh bone marrow samples, at the time of diagnosis to identify the leukemia-associated immunophenotypes (LAIPs), and in follow-up samples to detect MRD. Twenty two surface antigens in the following combinations: CD7/CD33/CD3/CD2/CD34/CD45, CD5/CD11b/CD19/CD10/CD34/CD45, CD15/CD22/CD20/CD117/CD34/CD45, CD64/CD34/HLA-DR/CD13/CD14/CD45 and CD3/CD56/CD4/CD16/CD8/CD45 were analyzed using combinations of fluorescein isothiocyanate-, phycoerythrin-, peridinin-chlorophyll protein-Cy5.5-, phycoerythrincyanin 7-, allophycocyanin-, allophycocyanin-H7 labelled monoclonal antibodies (BD Biosciences). For LAIP identification and MRD detection, 20.000 and 250.000 events were respectively acquired by a FACSCanto II cytometer (BD Biosciences),

and data were analysed with the FACSDiva software (BD Biosciences). ROC curve analysis was performed to establish the MRD cut-off, yielding the best segregation of AML patients into categories of risk for relapse at the different time points. *Results.* A total of 63 LAIPs were detected in 38 patients (91%) (M/F: 22/16; mean age: 50); morphologic CR was achieved after one cycle of induction therapy in 30 of them (79%) and after two cycles in the remaining 8 (21%). The most frequent aberrations of these patients were: asynchronous antigen expression (n:22; 35%), lineage infidelity (n:20; 32%), lack of antigen expression (n:11; 17%) and overexpression (n:10; 16%) (see table). LAIPs most frequently obtained were CD33+CD4+ (29%) and CD33+HLADR- (26%). After a median follow-up of 9 months (range: 5-14) 9 of 38 patients (24%) relapsed. ROC analysis showed that a MRD cut-off, set at 0.040% cells after consolidation therapy, allowed to identify 6 MRD-positive patients out of 7 relapsed patients with a sensitivity of 85.71% (95% CI: 42.13%-99.64%) and 11 MRD-negative patients out of 23 relapse-free patients with a specificity of 47.83% (95% CI: 26.82%-69.41%). The proposed cut-off was consistent with the most recent literature reporting an optimal value of 0.035%; indeed, a re-analysis of our data using this cut-off lead to the same results. *Conclusions.* MRD could be found by MFC in the vast majority of our AML patients and a level of 0.04% after consolidation correlated with subsequent relapse with acceptable sensitivity and specificity. This cut-off value was close to that reported in the literature. Further follow-up will clarify if MFC will be clinically useful.

Table 1.

	N° of cases
Lineage infidelity	
CD34+/CD7+	7
CD34+/CD19+	1
CD33+/CD4+	11
CD33+/CD2+	1
Asynchronous antigen expression	
CD34+/CD14+	2
CD34+/CD15+	1
CD34+/CD64+	1
CD34+/CD56+	2
CD117+/CD14+	3
CD117+/CD15+	1
CD117+/CD64+	6
CD117+/CD56+	3
CD33+/CD56+	3
Lack of antigen expression	
CD33+/HLADR-	10
CD33+/CD13-	1
Overexpression	
CD34++	4
CD117++	1
CD33++	2
HLADR++	3

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RAPID, ONE-STEP DETECTION OF BCR-ABL P210, P190 FUSION TRANSCRIPTS AND CONTROL GENE DIRECTLY ON RNA WITH A MULTIPLEX, ISOTHERMAL LAMP REACTION (RT-LAMP)

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Introduction. The molecular detection of the BCR-ABL fusion transcript is necessary for the genetic confirmation of Chronic Myeloid Leukemia (CML) diagnosis and for the risk classification of Acute Lymphoblastic Leukemia (ALL). *Methods.* LAMP is an isothermal reaction for rapid amplification of molecular targets. We developed a triplex RT-LAMP reaction for identification of both BCR-ABL p190 and p210 fusion transcripts and of the endogenous GUSb mRNA in a multiplex, close-tube format. GUSb amplification allows validation of negative results as an internal control of RNA quality and reaction conditions. The reaction is performed in a single tube directly on RNA thanks to the employment of a polymerase with both retro-transcription and amplification activities, thus reducing contamination risks. Furthermore, the reaction fluorescence real-time monitoring allows to avoid gel separation, thus diminishing set-up complexity and time-to-result. *Results.* The fluorescent RT-LAMP detects p210 (from K-562) and p190 (from TOM-1) RNA diluted in wild type RNA (from HL-60) down to 10-5 and 10-4 respectively. The assay specificity was tested on seven wild type cell lines: no false positive results was obtained on 121 replicates within the reaction time (50 minutes). Moreover, we validated this fluorescent RT-LAMP assay on RNA from patients affected by CML (n=30) and B-ALL (n=32) previously tested by RT-PCR. RT-LAMP detected the BCR-ABL transcripts, distinguishing between p210 (30 CML, 3 B-ALL) and p190 (29 B-ALL) and showing 100% concordance with conventional analysis. Finally, 22 RNA samples from patients affected by Philadelphia negative diseases (8 B-CLL; 4 AML; 2 ALL; 1 PV; 2 HES; 2 TE; 3 CLL) and 30 RNA samples obtained from healthy donors were tested. In these samples RT-LAMP amplified exclusively housekeeping gene transcript (GUSb). *Conclusions.* This novel fluorescent RT-LAMP assay allows a rapid and sensitive detection of both major and minor BCR-ABL fusion transcripts in a multiplex reaction with internal control. Triplex RT-LAMP is performed directly on RNA and monitored in real-time thus reducing the contamination risks of conventional multi-step procedures. It is highly specific, sensitive and reliable for diagnosis of Ph-positive diseases

PO-183

UNCX: A NOVEL HOMEOBOX GENE IN LEUKEMIA

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Introduction. The clustered homeobox (HOX) genes play a pivotal role both in normal and malignant hematopoiesis. A large number of AML cases show dysregulation of HOX genes due to their specific rearrangements or to the expression of fusion proteins involving MLL. We identified a AML M5 case with a t(7;10)(p22;p14) as the sole cytogenetic anomaly. The homeobox gene UNCX (7p22), silenced in normal hematopoietic tissues, was shown to undergo ectopic expression in the bone marrow of the patient, because of its translocation within the coding sequence of the housekeeping gene UPF2 (10p14). UNCX encodes a transcription factor involved in somitogenesis and neurogenesis, specifically expressed in the retina. Interestingly, two additional ESTs for this gene were mapped in its second intron, suggesting the occurrence of possible alternative transcripts not yet reported. We then decided to investigate the UNCX expression pattern in further AML cases as well as in hematological malignancies cell lines, negative for the t(7;10) rearrangement. *Methods.* Appropriate BAC probes were selected according to the UCSC Human Genome Browser (GRCh37/hg19), and used in FISH experiments to detect UNCX rearrangements. SNP Array CGH (Affymetrix 6.0) was also performed. RT-PCR and qPCR analyses were performed to investigate any dysregulation in UNCX expression pattern, using the retinoblastoma Y79 cell line and total brain RNA as positive controls. As UNCX is located within a large CpG island, its methylation status was evaluated by means of Mass Array Sequenom. *Results.* The methylation analysis of the CpG island at UNCX locus did not reveal any difference between the studied case and other AML patients negative for the rearrangement. Apart from the one harboring the t(7;10) translocation, two additional AML patients (one M1 and one M3 subtypes), out of 24, disclosed the same ectopic expression of UNCX. Among the analyzed leukemia cell lines, the chronic myeloid MEG-01 cell line was positive for UNCX expression. Surprisingly, this cell line was also shown to express a novel transcript variant including a portion of the second intron, already included in one mapped EST, also observed in Y79. In silico translation of this transcript isoform predicted the coding of a truncated UNCX protein, with a partial loss of the homeodomain. *Conclusions.* We described a new translocation event in AML, generating the ectopic expression of UNCX, never identified before in leukemia. This activation might be explained as a consequence of a position effect, due to the juxtaposition of UPF2 regulatory sequences to the UNCX gene. Due to its activation in additional AML cases and in the MEG01 leukemia cell line, without being accompanied by the t(7;10)

translocation, we suggest a role, although still to be elucidated, for UNCX isoforms in leukemogenesis. We plan to confirm this result on a larger cohort of AML cases, as well as to perform functional studies in order to clarify the UNCX role in leukemia.

PO-184

HIGH-RESOLUTION MELTING (HRM) ANALYSIS FOR RAPID DETECTION OF TET2 MUTATIONS IN MYELODYSPLASTIC SYNDROME (MDS)

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Introduction. Acquired somatic loss-of-function mutations of the TET2 gene have been frequently reported in MDS (range 10-20%). TET2 may act as tumor-suppressor gene able to modify the epigenetic state of DNA. We investigated TET2 mutations in 76 newly diagnosed MDS cases enrolled in an Italian multicentre prospective study (O-MDS-protocol, clinicaltrial.gov NCT01291745), using a novel HRM-PCR assay associated with sequencing analysis. *Methods.* Genomic DNA was extracted from bone marrow aspirates and buccal cell samples at diagnosis. HRM-PCR was performed on the 7900ABI PRISM (Applied Biosystems). HRM primers were designed based on the TET2 gene locus (Genbank NM_001127208). Amplified samples showing aberrant melting curves compared to wild-type (WT) were directly sequenced from a 1/10 dilution of the HRM product. Results were compared with direct genomic sequencing. All potential mutations were compared with published SNP data (dbSNP; http://www.ncbi.nlm.nih.gov/project/SNP) and annotated according to the coding sequence using of the TET2 isoform A. *Results.* A total of 10 templates were screened for known mutations to optimize the HRM analysis for each exon, variations in the melting curve were consistent with aberrations identified from direct sequencing. Subsequently, 66 previously sequenced patients were blindly subjected to HRM screening. Sequencing of buccal gDNA was used to confirm acquired mutations. Of the 66 pts, we observed one previously described mutation and 8 novel mutations of the TET2 coding sequence consisting of 5 InDel mutations, 2 aminoacid substitutions (Ser820Gly, Met1028Ile) and one substitution produced a STOP codon likely to alter or abrogate TET2 protein function. Seven mutations affected the coding region of ex3 and one the coding region of ex6. Patients with SNPs also displayed an aberrant melting curve thus allowing the identification of 53 previously described and annotated SNPs in 76 (69.7%) MDS patients. Notably, 2 mutations fell within the highly conserved LCX1. Of 53 SNPs identified, the individual SNP frequencies reflect those of the general population. Our incidence of detectable TET2 mutations is 11.8%. In silico analysis with PolyPhen-2 software (Adzhubei et al, Nat Methods 2010:7:248-249) predicted that the novel Ser820Gly mutation as benign, while the Met1028Ile variant is possibly damaging for TET2 protein function. *Conclusions.* HRM analysis proved a useful screening tool for genetic mutations in the TET2 gene coding region in MDS. Advantages of HRM-based screening, compared to traditional direct sequencing, include rapid evaluation of the extensive coding sequence of TET2 (9796bp), direct sequencing of the generated amplicon with no need of additional DNA, saving time and costs, and 100% correlation with direct sequencing protocols. Using this approach we identified 8 new heterozygous TET2 mutations HRM analysis is useful for high throughput assays to detect unknown mutations within the TET2 coding sequence.

PO-185

HIGH MSI2 GENE EXPRESSION IN BLASTIC PHASE OF CHRONIC MYELOID LEUKEMIA WITH NUP98/HOXA13 FUSION GENE

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Introduction. Ito *et al*. (Nature 2010) and Kharas et al. (Nature Medicine 2010) showed that Nup98/HoxA9 binds the Msi2 transcription start site, leading to Msi2 over-expression, downstream NUMB-NOTCH1 pathway deregulation, loss of myeloid differentiation and increased proliferation. HOXA9 is the best characterized NUP98 partner from a t(7;11)(p15;p15) originally identified in AML (Nakamura et al Nature Genetics 1996) and, subsequently, in cooperation with t(9;22)(q34;q11) in CML (Moore et al. Ann.N.Y.Acad.Sci. 2007). However, at least other 27 partners, including homeobox and also non-homeobox genes, have been to date identified in hematopoietic malignancies. Methods and Patients. Conventional cytogenetics and FISH were performed on peripheral blood cells at diagnosis of BC-CML in a 39-year old man; karyotype was 46,XY,t(7;11)(p15;p15),t(9;22)(q34;q11). FISH confirmed t(9;22)(q34;q11) and, given the 11p15 breakpoint, a break-apart assay was performed for NUP98 as previously described (Gorello et al Haematologica 2008). The 7p15 breakpoint was investigated with probes RP1-170O19 and RP1-167F23 spanning the HOXA cluster. Specific nested-PCRs used NUP98 forward and reverse primers for all cluster genes HOXA-9, -11 and -13. qRT-PCR was performed on patient's RNA from cryopreserved bone marrow with TaqMan probes (Applied Biosystems) Hs00292670_m1, for MSI2, and Hs00245445_m1 for the endogenous reference control, ABL1. Additional samples included 13 BC-CML, 12 CP-CML, 11 AML (3 NPM+FLT3+, 3 NPM+FLT3- and 5 PML/RAR FLT3+) and 3 non-neoplastic samples as controls. Expression levels were normalized with the Universal Human Reference RNA (Stratagene). *Results.* Three splicing variants of the in frame fusion transcript NUP98/HOXA13 were identified, all with the breakpoint between exon 16 (nt 2322) of NUP98 and exon 2 (nt 952) of HOXA13 gene. MSI2 was over-expressed in the NUP98/HOXA13 patient (6.21), another BC-CML case with complex karyotype (5.27), and 2/11 AML cases, both NPM+FLT3+ (6.69 and 6.92). Median values were: 1.96 for BC-CML, 1.78 for CP-CML and 3.55 for controls. *Conclusions.* We show for the first time that, in addition to NUP98/HOXA9, the NUP98/HOXA13 fusion also underlies MUSASHI2 over-expression in BC-CML. As MSI2 was over-expressed in another BC-CML case with no evidence of t(7;11), we are now investigating molecular lesions in this case. MSI2 over-expression in two NPM+FLT3+ AML confirmed previous results in this subset of AML (Griner et al. Cancer Biology and Therapy 2010). High MSI2 expression is confirmed as a consistent event in acute myeloid proliferations including BC progression of Ph- positive CML. Acknowledgements. FCRP: 2012.0108.021.

PO-186

QUANTIFICATION OF JAK2WT AND JAK2V617F ALLELE IN MRNA FROM PH(-) MYELOPROLIFERATIVE NEOPLASMS PATIENTS: CORRELATION WITH DNA ALLELE BURDEN AND DESEASE PROGRESSION

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Introduction. Currently, the JAK2V617F allele quantification in Ph- MPN is performed on genomic DNA. However, compared to DNA, mRNA analysis may offer some advantages such as: 1)higher sensitivity; 2) inclusion of platelet mRNA; 3) mRNA transcriptional functionality. Therefore, we developed an absolute allele-specific RQ-PCR method to quantify JAK2WT, V617F and ABL (as housekeeping gene) transcript levels aiming to: 1) verify specificity and sensitivity of this assay; 2)evaluate whether gene expression levels correlate with disease phenotype and may mark disease progression (i.e. ET toward PV). METHODS: To construct reference curves, including five 10-fold serial dilutions, two plasmid standards were manufactured to contain 150 bp of either JAK2WT and JAK2V617F cDNA sequence. To perform an allelic discrimination we used primers described by Merker et al (JMD, 2010): reverse primers are complementary to the WT or the mutant cDNA sequence, respectively. Assay sensitivity was determined by serial 10-fold dilutions of the K562 (JAK2WT) and HEL (V671F homo) cell lines which were included in each experiment as negative and positive controls. RESULTS: We tested cDNA synthesized from peripheral blood buffy coat specimens of 48 MPN patients (16 PV; 25 ET, 7 PMF), 13 patients with secondary polycythemia (SP) and 23 healthy donors (CTRL). Respect to the qualitative PCR method (Baxter el al, Lancet 2005) our RQ-PCR assay shows a 100% concordance rate in identify-

ing the presence of the JAK2V617F. V617F mutation was detected in 16/16 PV (100%), 19/25 ET (76%) and 5/7 (71%) PMF samples. The mean expression of JAK2WT allele, as absolute averaged copies+SEM was: healthy donors 12127+2181; SP 12244+2489; PV 4955+1642, WT ET 10876+1781; V617F ET 5693+830 and V617F PMF 3004+1442. Statistically significant differences were detected between CTRL and PV ($P<0.005$) and CTRL and V617F ET ($p<0.005$). The mean expression of V617F allele was PV 27107+12699, ET 6524 +1877 and PMF 29464+14587. A significant difference was found comparing PV and ET ($P<0.005$) and ET and PMF ($P<0.005$). Respect to the allele burden quantitative assay (IPSOGEN), the mutational burden (V617F/(V617F+WT) ratio detected by our assay is significantly higher (59% vs 36% burden in RQ-PCR and IPSOGEN, respectively; $p<0.005$). Three ET patients progressed to PV phase. The sequential analysis of the mutational burden ratio at diagnosis and during disease progression showed an increase in one patient's sample at both DNA and mRNA level. By contrast, in the remaining 2 cases the ratio remained constant at the DNA level, but significantly increased when the mRNA was analysed. *Conclusion.* Present data demonstrated that our method is as specific but more sensitive than the allele burden determination. In addition, the complementary determination of mRNA transcript levels may help in elucidate the pathogenetic mechanisms of the Ph- MPN diseases.

PO-187

PREVALENCE OF HHV-6 CHROMOSOMAL INTEGRATION (CIHHV-6) IN THE SEMEN OF MEN OBSERVED FOR SUSPECTED INFERTILITY

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Introduction. The phenomenon of HHV-6 chromosomal integration (CIHHV-6) is a unique form of HHV-6 latency, characterized by integration of apparently whole-genome length HHV-6 sequences into host chromosomes and transmission in a Mendelian manner. CIHHV-6 has a prevalence ranging from 0.21% to 3% either in healthy subjects or in transplant and leukemic patients from different geographical areas. Infertility, defined as the inability to conceive after 1 year of unprotected intercourse, is a problem that affects almost 15%-20% of couples in European countries. A few studies have so far investigated the prevalence of HHV-6 infection in the semen of infertility male patients. HHV-6 DNA sequences have been detected in 3.7% to 70% of semen samples, without adversely affecting semen parameters. From these studies, it has been argued that genital tract HHV-6 infection may not contribute significantly to male infertility. However, the issue of CIHHV-6 has so far not been addressed in this specific clinical setting. *Methods.* Semen samples have been prospectively collected from 238 adult men, undergoing evaluation for infertility. Samples were subjected to semen parameters analysis within 1 hour from collection, and then processed for freezing within 2 hours from collection. Molecular analyses, either a nested qualitative PCR assay or a quantitative real-time PCR assay (Nanogen advanced diagnostic kit), to amplify HHV-6 DNA sequences, have been performed on DNA, extracted from spermatozoa separated from seminal fluid by high-speed centrifugation. *Results.* The nested qualitative PCR assay documented HHV-6 DNA sequences in 41 of 238 (17.2%) frozen spermatozoa samples from men evaluated for suspected infertility. On the contrary, the quantitative real-time PCR assay resulted positive in 29 of 238 samples (12.1%), with HHV-6 DNA copy numbers ranging from 1 to 121,850 copies/100,000 cells. Among the HHV-6-positive sperm samples, semen parameters analysis documented leukocytospermia and bacteria in 69.4% and 33.3% of cases, respectively. Moreover, 50% of HHV-6-positive samples were either oligozoospermic or asthenozoospermic or teratozoospermic. Of interest, in 5 of the 238 individuals of our series (2.1%), the high HHV-6 DNA copy numbers were consistent with CIHHV-6. In 3 (75%) of the 4 subjects for whom semen parameters were available, abnormal sperm concentration and motility were observed. *Conclusions.* We have documented CIHHV-6 in 2.1% of semen samples collected from adult men, undergoing evaluation for infertility. Little is known about CIHHV-6 and pregnancy, but no published study has suggested to avoid pregnancy. We are evaluating the clinical consequences of assisted fertilization from individuals carrying CIHHV-6, and we would like to follow-up such pregnancies and the births, with particular interest in evaluating the physical and psychological development of children.

PO-188

CHROMOSOME 3 REORGANIZATION RESEMBLING CHROMOTHRIPSIS IN MDS

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Introduction. A number of cytogenetic abnormalities have been identified in myelodysplastic syndromes (MDS). Large deletions [such as del(5q)], as well as whole-chromosome aneuploidies (-7, +8), are the most common, whereas balanced rearrangements are rarely observed. We here report about a MDS case showing a multiple rearrangement involving one chromosome 3. The complexity of the alterations resembles what has been recently identified as chromothripsis, i.e. an extreme chromosome reorganization involving one or few chromosome. *Methods.* The fine structure of the derivative chromosome 3 [der(3)] was investigated by both FISH and SNP array CGH (Affymetrix 6.0 platform). The main genes involved in the composite rearrangement were subsequently tested for alteration in their expression pattern by means of immunohistochemical assays. The whole-genome sequencing of the bone marrow genomic DNA (by means a Illumina HiSeq2000 platform) is presently in progress. *Results.* FISH analyses mapped at least four breakpoints on the der(3). The rearrangements lead to the juxtaposition, in the same transcriptional orientation, of the 5' UTR of the non-coding RNA LOC100302640 gene to the whole coding sequence and 3'UTR of the forkhead box P1 (FOXP1) gene, resulting in its overexpression, as documented at protein level. Moreover, other two breakpoints were mapped within the non-coding RNA LOC344595 and TP63 genes. Interestingly, the latter showed a higher immunostaining activity for the p63TA isoforms in the blast elements of the patient, if compared to other myeloid malignancies samples. Conversely, no reactivity for the p40 (N) TP63 isoforms was detected in the patient's sample. *Conclusion.* Chromothripsis is a phenomenon by which one or few chromosomes are shattered and then stitched together in a different order via a single devastating event. To the best of our knowledge, the one reported here is the first case of MDS harboring a chromothripsis-like rearrangement. Particularly, the chatastrophic event disrupts FOXP1 and TP63, altering their bone marrow expression pattern. FOXP1, belonging to the P subfamily of the forkhead box (FOX) transcription factors, plays important roles in the regulation of tissue- and cell type-specific gene transcription during both development and adulthood. Chimeric genes involving FOXP1 gene are already described in hematological malignancies, although never deriving by an intra-chromosome 3 rearrangement. Similarly, TP63 gene expression alterations have never been reported before in myeloid neoplasias and their biological impact has to be clarified. We might speculate that both these two mutations might represent the driving forces for the selection of the bone marrow cell clone harboring the chromotripsis of chromosome 3. In the near future, the massive sequencing data will help us to define the multiple rearrangement at nucleotide level, perhaps disclosing further criptic gene/s alteration with a potential impact in leukemogenesis.

PO-189

NEW FUSION GENE INVOLVING EWSR1 IN ACUTE MYELOID LEUKEMIA

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Introduction. Different member of the TET-family genes, such as FUS, EWSR1, and TAF15, have been reported as fused to different transcription factors in various malignancies including Ewing's sarcoma, primitive neuroectodermal tumors, and acute myeloid leukemia. In leukemia, EWSR1 has been reported as involved in chromosomal aberrations only

in one case of an acute undifferentiated leukemia with a t(12;22)(p13;q12). In this case, the translocation caused the genesis of a chimeric transcript through an in-frame fusion between exon 7 of EWSR1 and exon 2 of CIZ1, a gene encoding a Zinc finger protein transcription factor. This fusion is thought to deregulate the expression of CIZ1 and mediate the oncogenesis through a not yet defined molecular mechanism. We report here a case of acute myeloid leukemia (AML) showing an amplification of a rearranged EWSR1 gene within a marker chromosome, originated by a complex rearrangement of chromosome 22. *Methods.* M-FISH and FISH experiments with appropriate WCP and BAC probes, selected by browsing the UCSC Human Genome Browser (http://genome.ucsc.edu/), March 2009 (GRCh37/hg19) release, have been performed to characterize the marker chromosome, detected as the main chromosomal anomaly by G-banding analysis. SNP array experiments have been performed to identify the amplified regions (in progress). *Results.* The observed marker chromosome was composed by alternatively repeated chromosome 19 and 22, fused to a seemingly normal chromosome 11q. FISH experiments with probes specifically designed for genes recurrently involved in leukemias, located respectively on chromosome 11, 19, and 22, revealed an amplified 5' portion of EWSR1. Conversely, no amplification was detected for the 3' portion of the gene. The involvement of ZNF444, a gene already reported as a fusion partner in a case of myoepithelioma with a t(19;22)(q13;q12), was excluded here by FISH. SNP array analysis (Affymetrix SNP 6.0 array), aiming at identifying amplified genes potentially fused to EWSR1, is in progress. *Conclusions.* To the best of our knowledge, the amplification of EWSR1 was never reported in AML. Furthermore, we present a novel amplified, likely chimeric, gene involving EWSR1 and a new chromosome 19 gene entity, that will be identified in the near future by means of SNP array and RACE-PCR experiments. The results obtained underline the involvement of TET-protein fusions in AML and support a crucial role for EWSR1 in myeloid leukemogenesis.

PO-190

INV(2)(P23Q13)-RANBP2/ALK REARRANGEMENT IN A CASE OF PEDIATRIC AML-M4

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Introduction. In the pathogenesis of haematological malignancies at least 3 Nucleoporin genes (RANBP2/2q13, NUP98/11p15, NUP214/9q34) produce abnormal fusion proteins as a consequence of diverse chromosomal rearrangements. Fusion between RAN-binding protein 2 (RANBP2),a nuclear pore complex protein and ALK, a receptor tyrosine kinase, resulted from inv(2)(p23q13) or t(2;2)(p23;q11~13) in 3 children with diverse myeloid neoplasms (Röttgers S et al., Leukemia, 2010). METHODS Case history. A 14 year old girl was admitted to the Mother and Child Health Care Institute, Belgrade, in May 2009 because of high white blood cell count (181.0 x103/cm3; neutrophils 45.6%, lymphocytes 6.9%, monocytes 47.5%), thrombocytopenia (22x109/cm^3), vomiting and abdominal pain. Bone marrow morphology indicated AML-M4. Karyotype was 46,XX,inv(2)(p23q13). The AML-BFM 98 protocol chemotherapy was started. Relapse in September 2009 with a karyotypic evolution, 45,XX,inv(2)(p21q11),-7[100%] was treated with FLG-Ida chemotherapy. The patient was referred to the Pediatric Onco-Hematology Unit, Perugia University in November 2009 for haploidentical stem cell transplantation. Before transplantation bone marrow karyotype had evolved to 46,XX,inv(2)(p23q11.2),-7,+mar[100%]. Rescue therapy (Myelotarg and Fludarabine) failed. In February 2010 CD34+ cells from a HLA haploidentical donor were infused. Because of relapse in April 2010 Donor Lymphocyte Infusion was administered. In November 2010 relapse recurred. Treatment with Etoposide and Interleukin 2 failed. The patient developed aspergillosis and died in March 2011. FISH. Putative RANBP2 involvement in inv(2)(p23q13) was tested in a FISH assay using genomic clone RP11-348G16 (encompassing the gene). ALK gene was investigated with Vysis LSI ALK Dual Color, Break Apart Rearrangement Probe. *Results.* Breakapart FISH assays for ALK gene indicated it was involved in inv(2)(p23q13) showing one green and one red signal split on inv(2). RANBP2 FISH test also indicated the rearrangement of the gene as a splitting of the probe RP11-348G16 was observed on inv(2). Unfortunately there was no biological material left to clone the predicted RANBP2/ALK fusion. *Conclusions.* This is the 4th case of a pediatric myeloid neoplasm associated with inv(2) (p23;q13)-t(2;2)(p23;q11~13)/RANBP2/ALK rearrangement. All patients shared haematological findings, such as myeloid phenotype and high white blood cell counts with excess of monocytes. All cases also showed monosomy 7. As reported for one of the other 3 previously described patients, the present case clearly confirmed -7 was a secondary karyotypic change during disease progression, indicating that the RANBP2/ALK rearrangement was the primary oncogenic event in this subset of pediatric myeloid leukemias. *Acknowledgment*. FCRP: 2012.0108.021.

PO-191

EMOCROMATOSI, BETA-TALASSEMIA E METABOLISMO DEL FERRO

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Introduction. Body iron homeostasis is safeguarded by cellular mechanisms that must be strictly regulated in order to satisfy erythropoietic needs and iron recycle requests. In the modulation of iron balance a critical role is played by the hemochromatosis gene, known as HFE, and by the hepcidin, a peptide produced by the liver. The first-one regulates the iron balance by forming complexes with: a) Transferrin Receptor 1 (TfR1), in case of iron deficiency or b) Transferrin Receptor 2 (TfR2), the TfR1 liver homolog, in case of iron overload. Hfe/TfR2 complex actives a signaling cascade resulting in the upregulation of hepcidin and, consequently, a decreased dietary iron uptake. Anemia and hypoxia downregulate hepcidin expression, increasing iron availability. When the iron balance is not properly maintained, several disorders and complications can appear. The hereditary hemochromatosis is a genetic disorder characterized by excess of iron absorption and, consequently, multi-organ failure. Progressive iron overload is also the most salient complication of -thalassemia. -thalassemia is an autosamal recessive disorder characterized by reduced rate of synthesis or no synthesis of -globin chains and by ineffective erythropoiesis. The blood picture of the patients with -thalassemia resembles iron deficiency and, in case of severe thalassemia, a blood transfusion regimen was the first measure effective in prolonging life. *Methods.* A clinical case was reported: Caucasian, 33 year-old female inhabitant in Basilicata (South Italy). The clinical signs are: fatigue, weakness, joint and abdominal pains and jaundiced complexion. The most important iron biochemical parameters were measured. After a step of DNA isolation form whole blood, the genetic test for hereditary hemochromatosis and beta-thalassemia were performed, by means of DNA amplification by PCR and hybridation of amplification products to a test strip containing allele-specific oligonucleotide probes immobilized as an array of parallel lines. *Results.* The result of the biochemical measurements are the following: transferrin 238± 16 mg/dL (normal range: 260-400 mg/dL); ferritin 922± 45 ng/mL (normal range: 10-100 ng/mL); serum iron 318± 11 µL/dL (normal range: 37-158 mcg/dL). CBC, HBV and HCV serum markers were negatives. GOT value was equal to 17± 5 UI/L (normal range: 0-40 U/L); GPT value was equal to 21± 8 UI/L (normal range: 0-40 UI/L). The genetic test results underlined that the patient is carrier of both hereditary hemochromatosis and -thalassemia. *Conclusions.* The patient is heterozygous for both diseases. The coexistence of these conditions alters the balance in iron homeostasis and exerts two competiting signals in modulation hepcidin expression: the increase in serum iron is unable to counteract anemia-induced down regulation of hepcidin gene expression and this aspect aggravates iron overload and its related pathological conditions.

PO-192

PROGNOSTIC RELEVANCE OF WT1 MOLECULAR LEVELS IN PATIENTS WITH ACUTE MYELOID LEUKEMIA AFTER ALLOGENEIC STEM CELL TRANSPLANTATION

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Introduction. WT1 is a panleukemic marker that is highly expressed in the majority of Acute Myeloid Leukemias (AML). Monitoring WT1 levels at different time points is very useful to assess disease status, particularly to detect minimal residual disease. The present study aimed to investigate the correlation between WT1 levels before transplantation and the risk of relapse in patients with AML after Allogeneic Stem Cell Transplantation (Allo-SCT). *Methods.* Since January 2010 to present 12 patients with AML underwent Allo-SCT in our Transplant Unit. Their WT1 molecular levels were monitored in Bone Marrow (BM) samples at different time points: at diagnosis, after induction therapy, before transplantation and 3, 6, 9 and 12 months after Allo-SCT. The samples have been analysed with Real Time PCR as described by Cilloni et al (JCO, 2009). Levels of WT1 are expressed as WT1 copies/ABL copies x 10^4. Normal range in bone marrow is less than 250 WT1 copies/ABL copies x 10^4. Numerical data are expressed as median (range). The Mann-Whitney U test was used to perform statistical analysis. *Results.* Our preliminary studies showed that molecular levels of WT1 in Bone Marrow samples are associated with leukemia burden. At diagnosis all patients had high levels of WT1, with a median value of 9948 WT1 copies/ABL copies x 10^4 (range 1325-18338). After induction therapy WT1 levels decreased in all patients. Patients who achieved a complete cytological remission showed a trend to lower levels of WT1 than those who had persistent disease (97; range 12-1878 vs. 2609; range 655 - 5868). Four patients relapsed after Allo-SCT (1, 3 and 6 months after). Molecular levels of WT1 at pre-transplantation time were higher in those patients who relapsed than in subjects who maintained complete remission (182; range 20-986 vs. 16; range 4-65; P=0.0242) (Fig ure1). At the time of relapse WT1 was highly expressed in BM samples in all 4 cases, with a median value of 6660 WT1copies/ABL copies x 10^4 (range 406-14257).*Conclusions.* In agreement with literature, our experience showed how WT1 molecular levels in BM samples correlate with leukemia burden. Furthermore, molecular levels of WT1 at pre-transplantation could be predictive of post-transplantation outcome and thereby useful for the modulation of immunosuppressive therapy based on patients' risk of relapse.

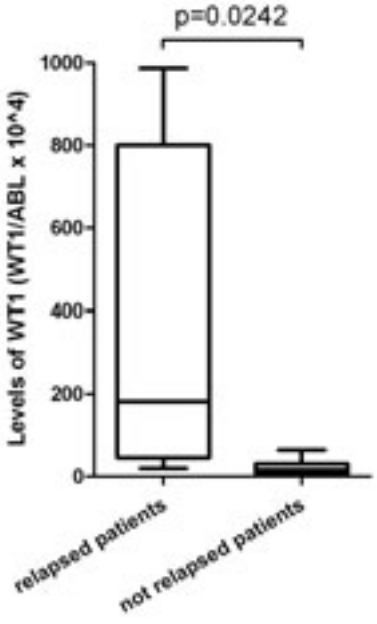

Figure 1. WT1 molecular levels at pre-transplantation time.

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MINIMAL RESIDUAL DISEASE IN ALLOGENEIC STEM CELL TRANSPLANTATION: A COMPARISON AMONG MULTIPARAMETRIC FLOW CYTOMETRY, WT1 EXPRESSION AND COMPLETE CHIMERISM STATUS.

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Introduction. Monitoring of minimal residual disease (MRD) is valuable for risk stratification and early detection of relapse in patients with acute leukemia (AL). The clinical utility of MRD has evolved with PCR amplification of leukemia- specific targets and Wilms' tumor gene (WT1),as well multiparametric flow cytometric (MFC) detection of leukemia – associated immunophenotypes (LAIPs). Highly sensitive monitoring of MRD is crucial after potential curative treatment as allogeneic stem cell transplantation (allo-SCT). Although the majority of previous studies performed chimerism analysis to evaluate the engraftment, some authors found an association between mixed chimerism (MC) and risk of relapse. With the purpose to use the complete chimerism (CC) status as a useful tool to detect MRD we subdivided CC patients in two study- categories (95-99% vs 100% of donor cells). Then, comparisons among CC, MFC and WT1 were performed in order to study three methods in monitoring patient's MRD after allo- SCT. *Methods.* Fresh BM samples from 25 patients (17 AML and 8 ALL) in both morphological CR and CC status after allo-SCT were investigated. MRD with MFC, WT1 expression and chimerism analysis was evaluated at different time points: +1, +3, +6 months after allo-SCT. The immunophenotypic analysis was performed using a six-color combinations and acquiring 250.000 events. RQ- PCR to test WT1 expression was made according to the standardized and quality-controlled method. Chimerism studies were performed with a multiplex amplification of 16 (STR). Differences in between techniques/methods variability were tested following Roy's approach. Results. A good agreement between MFC and WT1 was evidenced at each time point (P= 0.122 vs P= 0.910 vs P= 0.842, respectively)and overall (P= 0.345). Instead, no agreement between chimerism and MFC was statistically detected at all time points. Indeed, after one month from the transplant, 48% of patients showed a positive MRD by the MFC a front of 20% by the chimerism (P= 0.012). It was confirmed after six months (P=0.011) and in overall agreement (P<0.001). Similar results were observed from the comparison between WT1 and chimerism analysis. In particular, progressive greater disagreement occurred in order of proceeding time from the transplant (P=0.368 vs P=0.092 vs P= 0.021, respectively). Among all investigated clinical parameters, the donor type and the graft source seemed to be associated to the presence of minimal residual disease detected by the chimerism at different time points (P= 0.040 and P= 0.012 vs P=0.037 and P= 0.015, respectively). No significant statistical associations between clinical parameters and both MFC and WT1 were found. *Conclusions*. In our study, MFC and WT1 display comparable results in monitoring MRD after allo-SCT whereas complete chimerism status does not correlate with a complete clearance of blasts.

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QUANTITATIVE ASSESSMENT OF THE BCR-ABL P210 TRANSCRIPT IN BONE MARROW ASPIRATES USING GENE XPERT AUTOMATED PLATFORM

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Introduction. GeneXpert (Cepheid) is an automated system for the identification of the BCR-ABL gene fusion (p210). Briefly the system, that combines cartridge-based microfluidic sample preparation, starting from 200 l of peripheral blood, perform an RNA extraction, an RT-PCR and a nested PCR in less than 2 hours. GeneXpert provides a quantitative results aligned to the International Scale. The validated starting material is peripheral blood, no suggestions on the use of bone marrow is provided by the manufacturer. The aim of this study was to evaluate the

GeneXpert in the quantitative assessment of BCR-ABL transcript on bone marrow in CML patients. METHODS: Thirty-four samples of bone marrow aspirates were obtained from 17 CML patients under imatinib ot dasatinib therapy. Two aliquots for each sample (100 l and 20 l) were subjected to the preanalytical step of the Xpert Monitor Assay, within 24 hours from collection according to the manufacture's protocol. Briefly,100 L or 20 l of bone marrow for each sample aspirate were mixed with 40 L of proteinase K and incubated at room temperature for 1 minute. Subsequently 1 mL of lysis buffer was added and incubated at room temperature for 10 minutes. One thousand microliters of 100% ethanol was added, mixed and loaded into the cartridge. *Results.* The comparison between % BCR-ABL by GeneXpert on the two dilutions for each sample, was based on 17 patients corresponding to 34 samples. In 10 patients (59%), a slightly higher % BCR-ABL was observed in the 100 l dilution versus 20 L; in 5 patients (29,4%) a slightly higher % BCR-ABL was observed in the 20 l dilution versus 100 L. In 1 patient,(5,8%) there is a perfect correspondence between the two dilutions. In one case (5,8%) the comparison was not possible due to an invalid result caused by an high number of WBC (53600/L) in the 100 L dilution sample. To evaluate whether the differences in the quantity of transcript observed in the two dilutions could be relevant to determine the molecular response to treatment, we evaluate the % BCR-ABL considering the different response categories as recommended by the European Guidelines: CMR 4.0, CMR 4.5, CMR 5.0. Based on this evaluation in 13 patients (81,2%)100 L and 20 L resulted in the same level of molecular response. In three patients we observed a discrepancy between the two dilutions, with a lower response to the therapy observed in the highest dilution sample. *Conclusions.* The Xpert BCR-ABL Monitor Assay is a realiable method for detecting the BCR-ABL translocation in CML patients starting from bone marrow aspirates. In 81,2% of our patients there wasn't variability in the molecular response testing different BM dilutions, therefore we did not find useful to keep testing two different dilutions to monitor molecular response to treatment. In our hand 100 L BM dilution was chosen compared to 20 l to be analyzed used on the GeneXpert.

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PCM1/JAK2 FUSION FROM T(8;9)(P22;P24) TRANSLOCATION IN MYELODYSPLASTIC-MYELOPROLIFERATIVE NEOPLASIA

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Constitutively activated receptor tyrosine kinases (TK) induce cell survival and proliferation in Myeloproliferative Disorders (MPDs), not only in chronic myeloid leukemia (CML), which is associated with t(9;22)(q34;q11) and BCR-ABL1 fusion, but also in less frequent malignancies with an acquired reciprocal chromosome translocation involving FGFR1, PDGFRA or PDGFRB gene. JAK2 is a non-receptor tyrosine kinase that can also been involved in translocations in various hematological malignancies such as myeloproliferative-myelodysplastic diseases, AML and B-ALL. Here we report on a t(8;9)(p22;p24) in which JAK2 fuses to the human auto-antigen pericentriolar material (PCM1) gene in a 29 years-old man who was admitted to the Department of Hemathology in Parma because of leucocytosis (62.910/mm^3), mild anemia (12,2 gr/dL), and mild thrombocytopenia (129.000/mm^3). The white blood cell count showed N 61%, L7%, M5%, Meta 14%, Mielo 15%. LDH 593 U/L. Epo 17,7. Clinical examination revealed splenomegaly. The bone marrow biopsy exhibited hyperplasia/dysplasia of erythropoiesis with proerythroblasts and erythroblasts clustering in paratrabecular nest; diffuse fibrosis and eosinophilic hyperplasia. CD34 positive blast cells were not increased. Bone marrow aspirate confirmed an hyperplastic erythroid series with clusters of proerythroblasts and diserytropoiesis. Hyperplasia of myeloid precursors, decreased megakaryocytes and eosinophilia were also noticed. Karyotyping showed a t(8;9)(p21;p24) in 94% of metaphases. According to WHO classification diagnosis of MDS/MPS unclassifiable was made. FISH tests with genomic clones for genes known to be involved in Ph-negative chronic myeloid neoplasias i.e. PDGFRA/4q12, C-KIT/4q12, TET2/4q24 PDGFRB/5q33, FGFR1/8p11, ETV6/12p13, and for BCR-ABL1, were all normal. Break-apart FISH assays for JAK2/9p24, RP11-12510 (5'JAK2, green) and RP11-39K24 (3'JAK2, orange) and for PCM1/8p22, RP11-156K13 (5'PCM1, green) and RP11-484L21 (3'PCM1, orange), showed both genes were rearranged indicating the t(8;9)(p22;p24) underlay a PCM1-JAK2 fusion. The patient received cytoreductive therapy with hydroxyurea lasting for 10 days followed by IFN. Three months later cytogenetic analysis showed that no cytogenetic response was achieved and the patient was considered for allogenic stem cell transplantation. This case clearly shows that genetic analysis is mandatory in Ph-negative myeloproliferative disorders. Moreover, taking into account the present case and those already published, we suggest that, in addition to PDGFRA,PDGFRB and FGFR1, PCM1/JAK2 should be included as a genetic marker of the WHO 2008 subgroup "myeloid lymphoid neoplasms with eosinophilia".

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THE ISOTHERMAL LOOP MEDIATED AMPLIFICATION (LAMP) METHOD IS LESS AFFECTED BY AMPLIFICATION REACTION INHIBITORS IN RESPECT TO PCR

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Introduction. Several compounds can interfere with the nucleic acid amplification during polymerase chain reaction (PCR), leading to inhibition of reaction that can cause false negative or suboptimal results. PCR inhibitors originate from the starting material (i.e. heparin, haemoglobin, polysaccharides,..) or can derive from chemicals employed during the nucleic acid extraction procedure (i.e. phenol, ethanol, guanidium isothiocyanate,..). Their presence can be evaluated by determining the ratio of spectrophotometric adsorbance of the sample at 260 nm to that of 280nm or 230nm, to identify protein or chemical contaminations respectively. Samples with ratio values lower than 1.8 are considered not pure and are not effectively amplified by PCR. *Methods.* we have tested the susceptibility of the loop mediated amplification LAMP method to the presence of classical PCR inhibitors. The LAMP method evaluated was developed for the simultaneous qualitative detection of the BCR-ABL fusion transcripts RNA (both p190 and p210) and the GUSb mRNA as internal control. The assay detects the p210 and p190 targets with high sensitivity in clinical samples positive for BCR-ABL, while amplifies the GUSb RNA in wild type samples, validating negative results. *Results.* The effect of inhibitors on LAMP has been evaluated by testing 15 RNA samples extracted from BCR-ABL positive cell lines (K-562 and TOM-1) that artificially presented a high chemical contamination, being the ratio of spectrophotometric adsorbance at 260 nm versus 230 nm lower than 1 (min 0.34, max 0.90). Two RNA samples from healthy donors presenting a ratio 260/230 of 0.34 and 0.42 have been also evaluated. The problematic samples tested by LAMP have been detected with the same efficiency of high quality controls, without any delay in amplification time. Furthermore, two clinical samples (CML) contaminated by the presence of heparin have been tested by both Quantitative RT-PCR and LAMP. The Quantitative RT-PCR analysis was inhibited by the contaminant and was carried out successfully only after sample treatment with heparinase. Differently, LAMP detected the BCR-ABL p210 efficiently in 25 minutes. *Conclusions.* the isothermal LAMP method demonstrated to be significantly less affected by the compounds that commonly inhibit the PCR reactions. LAMP amplified efficiently RNA targets impure due to the presence of chemicals derived for extraction process. Moreover, tested on samples contaminated with heparine, LAMP correctly amplified the target RNA that PCR was not able to detect. These results suggest that LAMP is a reliable molecular method that allows to detect efficiently the bad quality samples, decreasing the risk of false negative results.

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