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XIII Congress of the Italian Society of Experimental Hematology

Rimini, Italy, October 15-17, 2014

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ABSTRACT BOOK

XIII Congress of the Italian Society of Experimental Hematology

Rimini, Italy, October 15-17, 2014

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XIII Congress of the Italian Society of Experimental Hematology

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MAIN PROGRAM

MESENCHYMAL STROMAL CELLS AND CANCER

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Mesenchymal stromal cells (MSCs) are multipotent non-hematopoietic stem cell precursors residing virtually in all pre-natal and adult tissues, including bone marrow (BM), and capable of self-renewal and differentiation into a number of mesodermal cell lineages (fibroblasts, osteoblasts, adipocytes, and chondrocytes). MSCs display a homogeneous mesenchymal immunophenotype (*i.e.* CD105, CD44, CD73, CD90, CD146 marker expression) and acquire, once activated by inflammatory cytokines (*licensing*), a strong and broad modulatory effect towards all the effector cells of both innate and adaptive immunity, a property that is shared also by the fibroblastic progeny. MSCs are essential components of the BM microenvironment regulating and supporting the survival, proliferation and differentiation of normal hematopoietic stem cells and progenitors, as well as the development of different tumors, including hematological malignancies. Senescence or genotypic abnormalities of BM-MSCs may determine phenotypic alterations of hematopoietic stem cells and progenitors that may eventually lead, if additional genotypic abnormalities occur, to the onset of myelodisplasia and leukemic transformation. In addition, inflammatory licensing of the mesenchymal progenitors may eventually contribute to tumor development by favouring the onset of a *'immunological sanctuary'* that prevents tumor cells from being eliminated by immune effector cells. Different molecular pathways are involved in stromal cell supportive functions towards cancer cells; among them, Notch signalling plays a significant role in the reciprocal interactions between stromal microenvironment and neoplastic cells of hematological origin, such as ALL, CLL and AML cells, by promoting cell survival and resistance to chemotherapy. Therefore, targeting Notch pathway activation may represent a useful strategy to overcome drug resistance and improve the efficacy of conventional treatments.

INFLAMMATORY AND IMMUNOREGULATORY PATHWAYS WITHIN BONE MARROW MICROENVIRONMENT

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Over the last few years, new biological insights have been provided supporting the notion that, along with tumor cell-autonomous defects, cell-extrinsic factors have a crucial role in leukemia generation and maintenance. In particular, inflammatory networks ignited within the bone marrow (BM) microenvironment appear to play a crucial role in leukemia initiation and progression, as well as in response to chemotherapy. Indeed, on the one hand, the hyper-activation of inflammatory networks has been indicated as a key contributor to tumor development. On the other hand, the release of abundant inflammatory mediators within the tumor microenvironment has been shown to trigger pro-inflammatory networks and enhance adaptive immune responses, promoting the presentation of tumor-associated antigens and attenuating tolerogenic pathways. During the breakdown in cellular physiology that accompanies tumor development, including leukemia, tumor cells acquire some properties, which are defined through the interaction with the host environment (cell-extrinsic). In particular, the immune system-tumor interaction plays a dual role in tumor development both by eliminating tumor cells and by facilitating tumor escape from immune control. The genetic basis of this process, called cancer immunoediting, remains poorly understood as well as its interplay with other aspects of malignant conversion, such as tumor cell proliferation and apoptosis. In that context, the immunological microenvironment seems to act as a fundamental background where cell-to-cell interactions and interplay may influence leukemia growth and response to chemotherapy. *Inflammatory pathways*. Hematopoietic Stem and Progenitors Cells (HSPCs) reside within the BM stem cell niche, a nurturing environment believed to protect HSPCs from external insults. Nonetheless, recent findings showed that HSPCs actively sense pro-inflammatory factors: TNF , Interferons, Toll-Like Receptors and extracellular nucleotides – so far believed to be active on terminally differentiated cells only – have been shown to modulate also HSPC functions. Here, the role of the Tissue Inhibitor of Metalloproteinases-1 (TIMP-1) in human hematopoiesis will be described. TIMP-1, member of the mammalian family of the Tissue Inhibitors of Metalloproteinases (TIMPs), is also a key member of the inflammatory milieu: TIMP-1 promoter is a direct target of NF-kB (Xia et al.) and its expression has been previously reported to be stimulated by pro-inflammatory cytokines, including IL-6, TNF-alpha, and IL-1beta. As a member of the TIMP family, TIMP-1 was initially described as an endogenous inhibitor of all MMPs and ADAM10. However, over the past years, several reports contributed to shed light on unsuspected cytokine-like functions of TIMP-1 and highlighted its role in multiple biological processes, including cell growth and survival. In the hematopoietic system, we found that TIMP-1$^{-/-}$ mice present a consistent decrease in BM cellularity, including LT-HSCs, and in the capability to sustain. The role of exogenous TIMP-1 in promoting HSPC expansion and the underlying molecular pathway will be presented. Our findings demonstrate that rhTIMP-1 acts as a direct regulator of human CD34+ HSPC proliferation. The molecular dissection of TIMP-1's signaling revealed that TIMP-1's cytokine functions in HSPCs are mediated by the tetraspaning receptor CD63 and involve the activation of PI3K/pAKT signaling pathway. These molecular events eventually lead to cyclin D1 upregulation, promoting cell cycle activation and proliferation. *Immunoregulatory pathways*. Indoleamine 2,3-dioxygenase (IDO) is a key enzyme in the tryptophan metabolism that catalyzes the initial rate-limiting step of tryptophan degradation along the kynurenine pathway. Tryptophan starvation by IDO consumption inhibits T cell activation, while products of tryptophan catabolism, such as kynurenine derivatives and O_2 free radicals, regulate T cell proliferation and survival. For these reasons, IDO has immunosuppressive activity. A wide variety of human solid tumors have been demonstrated to express an active IDO protein and transfecting IDO into tumor cells prevents their rejection by pre-immunized hosts. Also AML cells, and not normal HSCs, may constitutively express IDO, which, in turn, exerts its inhibitory effect on T-cell immunity by inducing the conversion of $CD4^+CD25^-$ into $CD4^+CD25^+$ T regulatory cells. The expression of IDO by AML blasts has been correlated with poor clinical outcome in terms of overall survival. IDO expression can be regarded as a novel mechanism of leukemia escape from immune control and its inhibition may represent a novel anti-leukemia therapeutic strategy. Indeed, for solid tumors, preliminary clinical trials are currently on-going testing the safety, and clinical potential, of novel compounds, acting as IDO-inhibitors.

LONG NON CODING RNAS: STRUCTURE, EXPRESSION AND ROLE IN NORMAL AND MALIGNANT HEMATOPOIESIS

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Whole genome and transcriptome sequencing technologies have shown that at least 60% of the mammalian genome is transcribed into RNA. Then, so-called "junk DNA" are now known to encode a large and varied population of noncoding RNAs (ncRNAs). Among these ncRNAs, the long non-coding RNAs (lncRNAs) are defined as RNAs consisting of more than 200 nucleotides and lacking an open reading frame of significant lenght.[1] LncRNAs are generally transcribed by RNA polymerase II, and can account for nearly 60% of all non-ribosomal and non-mitochondrial RNA in human cells. LncRNAs length ranges from from 200 nucleotides to 10 kilobases, have a 5' terminal methylguanosine cap and are usually spliced and polyadenylated. Alternate pathways also contribute to the generation of known lncRNAs, which include a contingent of non-polyadenylated lncRNAs likely expressed from RNA polymerease III promoters and lncRNAs that are excised during splicing and small nucleolar RNA production.[1] It is difficult to calculate the exact number of lncRNAs, with current lncRNA catalogs ranging between 5000 and 15000 transcripts. Whereas the number of known human protein-coding genes has remained stable over recent years, the number of known lncRNAs continues to increase, and lncRNAs may eventually rival protein-coding genes in number and diversity. Given their unexpected abundance, long non coding RNAs were initially thought to be spurious transcriptional noise resulting from low RNA polymerase fidelity. Subsequent studies revealed that lncRNAs affect gene expression through direct interaction with proteins, DNA, and/or protein-coding RNAs. In fact, several well-characterized lncRNAs have been reported to regulate gene expression through a variety of mechanisms: 1) by chromatin modification, recruiting chromatin remodeling complexes to specific gene loci, 2) by enhancing or inhibiting the transcription of their target genes. 3) by post-transcriptional processing, including RNA splicing, editing, transport, translation and degradation. Several subsequent results, combined with the observation that thousands of protein coding genes present antisense transcripts, have enforced the idea that antisense lncRNAs generally control the expression of their cognate protein-coding genes through epigenetic regulations.[2] This model has profound implication for our understanding of disease, particularly cancer, since dysregulation of lncRNA regulating the expression of a tumor suppressor or oncogene may be one of the hits that leads to oncogenesis.[3] Despite the increasing number of studies on lncRNA expression and lncRNA involvement in solid tumor formation, lncRNAs have not been extensively characterized in normal and malignant hematopoiesis.[4]. Recently Paralkar *et al.* examined the lncRNA expression in murine megakaryocyte-erythroid precursors (MEPs), MK and erythroblasts, as well as in human erythroblasts, defining hundreds of lncRNAs unique to each cell type.[5] Alvarez *et al.* found that the expression of lncRNAs is strictly related to a specific tissue or developmental stage.[6] They highlighted that lincRNAs EC-2, -4, and -9, are required for erythrocyte maturation in the fetal liver but are absent in the adult bone marrow. Recent reports have identified aberrant expression of several lncRNAs, such as WT1- as,[7] MEG3,[8] and CDKN2B-as (ANRIL),[9] in hematological malignancies; in this vein, our group investigated the expression of ANRIL, MEG3 and WT1-antisense lncRNAs in CD34+ cells from primary myelofibrosis (PMF) patients. The results evidenced that the majority of PMF samples displayed a co-upregulation of WT1 and its antisense RNA compared to controls. These samples also showed an increased MEG3 expression. In these patients, we found a correlation with WT1/WT1-as/MEG3 expression levels and high Dynamic International Prognostic Scoring System (DIPPS) plus score and elevated number of circulating CD34+ cells. Moreover, the expression pattern of CDKN2B/ANRIL distinguished a group of patients characterized by an upregulation of CDKN2B, and among these, a subgroup with downregulated ANRIL. Of note, this group of patients exhibited a high grade of BM fibrosis and the presence of JAK2V617F mutation.[10] Our results suggest that a deregulated expression of these lncRNAs could play a role in PMF pathogenesis and progression. As a whole, lncRNAs could be involved in the development of hematological neoplasms and their abnormal expression could be used in future as potential disease marker and/or therapeutical target.

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PAROXYSMAL NOCTURNAL HEMOGLOBINURIA

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Paroxysmal nocturnal hemoglobinuria (PNH) is characterized by complement-mediated intravascular hemolysis due to the lack of CD55 and CD59 on affected erythrocytes. The treatment of paroxysmal nocturnal hemoglobinuria (PNH) has dramatically changed since the availability of the first clinical complement inhibitor eculizumab. Eculizumab treatment has been proven terrifically effective in controlling intravascular hemolysis of PNH; however, unmet clinical needs are emerging. The anti-C5 antibody eculizumab has proven clinically effective, but uncontrolled C3 activation due to CD55 absence may result in opsonization of erythrocytes, possibly leading to clinically meaningful extravascular hemolysis. To date, there is an obvious unmet clinical need, because even with anti-C5 treatment strategies one third of patients remains transfusion-dependent, and an additional third still lives with moderate anemia. Indeed, starting from the anti-C5 therapy that is already in the clinic, novel approaches are investigating the possibility of intercepting the complement cascade upstream, at the level of C3 and C3 convertase or of other initiating factors. Upstream complement inhibition at the level of C3 or the C3 convertase is now considered a promising approach for alternative complement therapeutics. Different strategies are currently under investigation; so far, successful *in vitro* studies have been conducted with: i. an antibody against C3b (mAb 3E7); ii. the fusion protein TT30 that combines regulatory domains of FH with the iC3b/C3d-binding domains of CR2; iii. a novel engineered inhibitor that links the regulatory and surface recognition areas of FH (mini-FH); iv. small peptide inhibitors members of the compstatin family (which are the object of this application). Additional options are currently under development, aiming to investigate novel targets as well as novel methods of inhibition. To date, clinical translation plans have been started with the fusion protein TT30 only. However, pre-clinical data with other agents seem robust enough to support clinical translation with other second-generation complement inhibitors. This is the case of compstatin analogs, and in particular of the derivative AMY-101, which has shown *in vitro* a complete abrogation of intravascular hemolysis of PNH erythrocytes, as well as a full prevention of their C3 opsonization. This supports the concept that, by blocking early phases of complement activation, AMY-101 should result in a better control of both intravascular and extravascular hemolysis in PNH patients. Combined with the excellent bio-availability and the safety profile demonstrated in non-human primates, these data pave the way for forthcoming human studies. The second era of complement inhibition has started; hopefully it will lead to substantial improvements of current anti-complement treatment in PNH and in other complement-mediated diseases.

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CLONAL HETEROGENEITY AND STRATEGIES TO OVERCOME IT IN MULTIPLE MYELOMA

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MM is a clonal proliferation of malignant plasma cells. The genome of MM cells is remarkably complex with profoundly altered karyotypes including aneuploidies, chromosomal translocations, and frequent DNA copy-number variation. Recent large next-generation sequencing efforts have also identified somatic mutations affecting a limited set of genes with relatively high frequency, including established oncogenes and tumor suppressors as KRAS and NRAS, BRAF and p53 alongside genes not previously implicated in carcinogenesis such as DIS3 and FAM46C (Bolli *et al.*, 2014; Chapman *et al.*, 2011; Lohr *et al.*, 2014). A dismal finding of these studies have been the remarkable heterogeneity of MM tumor cells, with the existence of several subclones within the MM cell population, which would make any prospective pharmacological targeting problematic. Here we describe in detail these findings, we hypothesize possible causes, and finally propose possible synthetic lethal approaches to overcome the protean genomic anatomy of MM cancer cells. This strategy is centered on common features shared by the various subclones that constitute the cancer population, and not specific for each subclone, thus overcoming the limitations of targeted therapies aimed to mutated gene products. In detail, we argue that exploiting the ongoing, pervasive DNA damage that is present in multiple myeloma cells could represent the avenue to overcome MM cell heterogeneity. To this end, we propose two strategies. The first approach stems from our recent findings, that upon DNA damage MM cells activate a p53-independent, pro-apoptotic network centered on nuclear relocalization of the ABL1 kinase (Cottini *et al.*, 2014). Although nuclear ABL1 triggers cell death through its interaction with the Hippo pathway co-activator YAP1 in normal cells, we show that low YAP1 levels prevent nuclear ABL1-induced apoptosis in hematological tumor cells. YAP1 is under the control of a serine-threonine kinase, STK4. Importantly, genetic and pharmacological inactivation of STK4 restores YAP1 levels, triggering cell death, *in vitro* and *in vivo*. Our data therefore identify a novel synthetic-lethal therapeutic strategy to selectively target cancer cells with endogenous DNA damage and low YAP1 levels. A second approach exploits the reliance of MM cancer cells on physiological mechanisms that are essential to relieve pro-apoptotic mechanisms in MM cells. Additionally, we found that increasing the DNA damage on already stressed MM cells further increase MM cell death. In conclusion, we propose an integrated synthetic lethal approach that might provide a novel targeted treatment in MM patients with high heterogeneous subclonal architecture and currently refractory to established therapies.

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PATHOGENESIS OF THERAPY-RELATED MYELOID NEOPLASMS

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Therapy-related myeloid neoplasms (t-MN) are myeloid disorders, including acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS), developing in patients treated with radiotherapy and/or chemotherapy for a previous cancer or an autoimmune disease. Sporadic cancer is the result of a complex interplay between environmental exposures and many susceptibility alleles, each of which only gives a small contribution to overall risk. The scenario might be different in t-MN, in which only a minor, but not negligible, proportion of patients exposed to specific cytotoxic agents develop leukemia, so that susceptibility factors, specific or generic, have been hypothesized. In this line, cytotoxic therapy may induce chromosomal alterations and genetic mutations in hematopoietic progenitors leading to leukemogenesis in susceptible subjects.[1] Several studies have investigated the impact of the genetic background of the host on t-MN predisposition. Among these, polymorphisms and germline variants in the genes belonging to detoxification and DNA repair pathways were the first candidates accounting for t-MN predisposition. Cellular systems for defense from genotoxic chemotherapeutic and radiotherapeutic attacks rely on phase I and II detoxifi cation enzymes, involved in the activation, conjugation and elimination of carcinogens, and on DNA repair enzymes. In this line, when detoxification and/or DNA repair are ineffective, the DNA damage can cause chromosomal instability leading to severe failure of cell functions and/or apoptosis. As a consequence, polymorphisms in genes involved in detoxification, DNA repair and apoptosis may modify the individual risk to develop t-MN in patients treated with cytotoxic drugs. Recently, we have shown that single nucleotide polymorphisms (SNP) in genes implicated in the apoptosis pathway may play a relevant role as risk factor in t-MN. In particular, carriers of the BCL2L10-21Arg variant have a reduced risk of developing t-MN. In this line, we hypothesize that the missense polymorphisms in BCL2L10, one key regulator of apoptosis, could deregulate the entire apoptotic pathway, modifying the risk of therapy-related myeloid neoplasms.[2] Mutations of genes belonging to the Fanconi Anemia pathway may also act as predisposition alleles is selected cases.[3] Predisposition might be even more important in non-neoplastic diseases treated with chemotherapy, where susceptibility for a primary malignancy is not a confounding factor. In this line, homozygous variants of BRCA2 and XRCC5 DNA repair enzymes, particularly when combined, and a single nucleotide polymorphism in the promoter region of the detoxification enzyme CYP3A4 were significantly more frequent in patients with acute promyelocytic leukemia (APL) and multiple sclerosis treated with mitoxantrone, when compared to matched controls.[4] Molecular changes distinctive of t-MN include not only genetic, but also epigenetic changes, with hypermethylation of several tumor suppressor genes, due to the cytotoxic treatment, which may cause chromosomal damage very early, many years before the onset of the t-MN.[5] Telomere shortening as marker of reduced regenerative capacity of hematopoiet-

ic stem cells has been shown to be predictive of MDS development in lymphoma patients following high-dose therapy, and has been associated with reduced generation of committed progenitors.[6] On the other hand, somatic mutations in epigenetic and spliceosome enzymes have been shown to contribute to the pathogenesis of MDS, through deregulation of several tumor suppressor genes. We have recently shown that the incidence of mutations of epigenetic and spliceosome genes varies in t-MN according to the type of previous malignancy. They are frequent in t-MN following solid tumors, but rare or absent in patients with a previous lymphoproliferative disease.[7] Recently, new sequencing technologies have enabled large screening of somatic mutations in myeloid malignancies, leading to the discovery of new hot spot mutations in genes candidate for leukemic transformation. Among these, SET binding protein 1 (SETBP1) has been reported as frequently mutated in chronic myelomonocytic leukaemia, atypical chronic myeloid leukaemia, secondary acute myeloid leukaemia and in distinct subgroups of primary myelodysplastic syndromes, such as refractory anemia with excess of blasts.[8] Unexpectedly, frequency of SETBP1 point mutations in the SKI homologous domain was very low in t-MN patients.[9] The low frequency of SETBP1 mutation in t-MN, similar to most epigenetic and spliceosome mutations, may suggest that other major changes, such as complex and monosomal karyotypes, together with TP53 mutations, may play the major role in therapy-related leukemogenesis. TP53 mutations are present in about 20% of t-MN cases. In a preliminary report, TP53 mutations have been found in bone marrow samples several years before the onset of t-MN. The aberrant clone may gain a proliferative advantage over time and drive the evolution of the disease and the acquisition of additional karyotype abnormalities.[10] Data from the Italian registry of therapy-related neoplasms, including almost 300 patients, confirms that karyotype abnormalities are very frequent in t-MN, present in 66% of patients.[11] Significant factors associated to shorter latency between the primary treatment and the t-MN were the addition of Topoisomerase-II inhibitors to alkylating agents. Radiotherapy alone was associated to a very long latency. This raised the question whether these MN are really treatment-related or second tumors in patients who are cancer-susceptible. Prognosis of t-MN patients is poor, with a median survival of less than one year in most patients. The only curative option remains allogeneic stem cell transplantation, which is only feasible in a minority of younger patients. Since t-MN are iatrogenic malignancies ,prevention should be the final goal. Defining not only the susceptibility factors but also the early changes in hematopoietic stem cells should help to minimize t-MN risk in patients undergoing cytotoxic treatment.

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CAN WE CURE NON HODGKIN'S LYMPHOMA PATIENTS WITHOUT CHEMOTHERAPY?

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In the past decades, chemotherapy and radiation therapy have been the mainstream for Non Hodgkin lymphoma (NHL) treatment, achieving good results especially with drugs combination for indolent and aggressive lymphoma, with overall response rates of 70-80%. However, its drawbacks are represented by well-known immediate and delayed toxicity and substantial inefficacy to treat relapsed/refractory patients. In 1997, the approval of the first MoAb anti-CD20 Rituximab heralded the era of biologic drugs in the setting of B-cell malignancies. Over the last few years, several biological targeted drugs have been developed on the basis of new available insight in cell cycle, mechanisms of cancer transformation, interactions between cancer and environment (signal transduction, apoptosis, angiogenesis, immune system). Figure1.

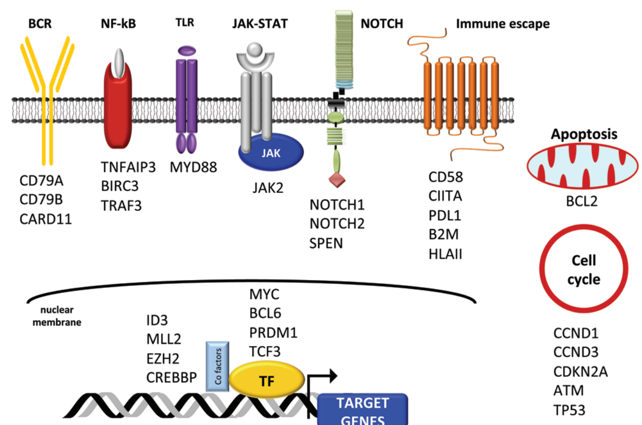

Figure 1. Deregulated pathways in B-NHL.

We reviewed available data in the field of sparing chemotherapy in front line treatment of NHL patients. Table 1. The only currently available chemo-free therapy for indolent and follicular lymphoma is rituximab, which in weekly induction doses demonstrating its activity with an overall response rate (ORR) and complete response (CR) of 80% and 28% respectively, with a PFS of approximately 23,5 months.[1] The RESORT randomized trial compared rituximab maintenance against rituximab retreatment, indicating a lack of significant benefit in terms of time to treatment failure for rituximab maintenance in this setting.[2] Currently, higher doses and different schedules of Rituximab are under evaluation especially for patients with a high tumor-burden and different pharmacodynamic profiles. Moreover, some new anti-CD20 (ofatumomab, obinotuzumab and veltuzumab) engineered to exert enhanced complement dependent cytotoxicity (CDC) or antibody dependent (ADCC) might be superior to rituximab (alone or in combination). Obinutuzumab has been recently approved in first line therapy in CLL/SLL in combination with chlorambucil.[3] Other strategies are investigating the combination of rituximab with other naked MoABs as for rituximab combined with Galiximab

(anti-CD80) in a phase II CALGB trial where 61 patients with previously untreated FL showed an ORR of 72.1% and a PFS of 2.9 years.[4] Moreover new perspectives are represented by antibody-drug conjugates (ADC) direct to CD19, CD22, CD79 that have shown activity in recent phase II clinical trials.[5] More recently a new class of MoAbs, called the checkpoint blockade inhibitors, mainly anti PD-1/PD-L1 are tested in ongoing phase 1-2 studies in B-NHL with encouraging signs that this strategy may be useful in the future.[6] The oral immunomodulatory agents (IMIDs) as lenalidomide appeared as another potentially active drug to be combined with rituximab. As a single agent, responses to lenalidomide were quite modest and observed in approximately 25% of patients. Hence, for indolent lymphoma, lenalidomide has been combined with rituximab (R-squared) as an innovative approach to treat patients with newly diagnosed advanced-stage indolent lymphoma which requiring therapy. Lenalidomide may potentiate rituximab-induced killing by enhancing immune synapse formations and NK cell activity, resulting in increased ADCC. In the front-line setting a combination of lenalidomide at a dosage of 20 mg/day for 21 out of 28 days, and monthly infusion of rituximab, for a total of 6 months has been evaluated in 110 patients with indolent lymphoma with an ORR of 86% and notably in the follicular lymphoma (FL) setting with CR of 94%.[7] This new chemotherapy-free regimen is now compared in a ongoing large phase III randomized trial (RELEVANCE) to a standard chemoimmunotherapy approach. Figure 2.

Table 1. Options for chemotherapy-free approaches currently evaluated in patients with B-NHL.

Agents and pathway	Drugs	Rationale and hypothesis	Current status of development
Anti CD20 MoAB	Ofatumomab Veltuzumab Obinotuzumab	Optimizing dose and scheme of Rituximab Enginnering more active anti CD20 Moabs	Clinical superiority as single agent not yet to be demonstrated in randomized studies
Naked Ab with different targets on B-cell	Anti-CD80 (Galiximab) Anti-CD22 (Epratuzumab) Anti- CD37 (Epratuzumab)	Should provide an anti-tumor effect if the target is well chosen	Limited efficacy as single agents and mostly investigated in combination with anti CD20
Antibody drug conjugates (ADC)	Polatuzumab vedotin (anti CD 22) Inotuzumab ozogamicin (anti CD22) Pinatuzumab vedotin (anti CD79 b)	Increase the anti lymhoma activity of specific Moabs with cytoxic drug conjugates	Good activity in recent phase II clinical trials
Immumodulatory agents	IMIDs (lenalidomide) Anti-PD1 (pidiluzumab, nivolumab) Anti-PDL1 Blinatumomab	Intrinsic activity and/or increasing effector cell activity in the presence of anti-CD20 antibodies Modulating effector cell activity	Encouraging preliminary results, risk/ benefit ratio to be determied in randomized trial
PI3K/AKT/mTOR signaling pathway and B-cell receptor pathway	PI3K inhibitors (Idelalisib, IPI-145, etc.) mTOR inhibitors (Temsirolimus) BTK-inhibitors (Ibrutinib) Syk-inhibitors (Fostanatinib	Modulating effector cell activity in B-cell malignancies Counteracting anti-apoptotic molecules	Phase II results encouraging Toxicity and low efficacy Preliminary signals of activity in phase II trial

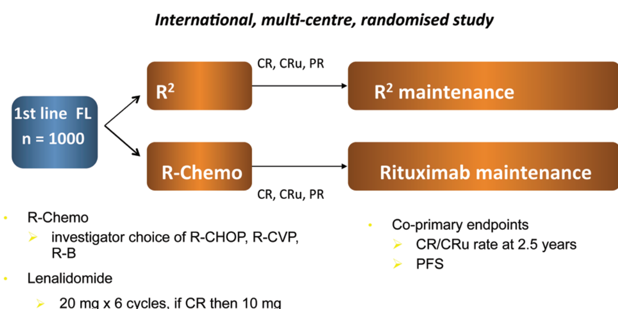

Figure 2. Flow chart of the randomized phase III trials RELEVACE.

The association of proteasome-inhibitor Bortezomib (already approved for refractory mantle cell lymphoma) with rituximab has been tested in indolent NHL showing an encouraging ORR and CR rate of 70% and 40% respectively with an overall survival of 87% at 4 years.[8] The B cell-receptor pattern offers numerous therapeutic targets, with drugs inhibiting BTK, Phosphatidylinositol 3-kinase (PI3K) and SYK. In particular, Ibrutinib, a potent irreversible inhibitor of BTK, has been granted breakthrough status by the US FDA for MCL and CLL. In a comparative study between ofatumumab and ibrutinib in patients with poor risk CLL/SLL Ibrutinib demonstrated an ORR of 63%.[9] However, it fails to eliminate the malignant clone in CLL. The main concern is the peculiar response to Ibrutinib, indeed 20% of patients with CLL have persistent lymphocytosis, and most patients are likely to have residual tumor cells, enabling the emergence of resistant subclones. The association with other drugs could mitigate this weakness. For example, an *in vitro* screening in ABC DLBCL suggested a favourable interaction of Ibrutinib with compounds including inhibitors of the PI3K-AKT-mammalian target of rapamycin signalling cascade, other B-cell receptor pathway inhibitors, and Bcl-2 family inhibitors.[10] Recently, first-in-class PI3K delta Idelalisib was approved for relapsed/refractory CLL/SLL and FL by FDA[11]. Given the enthusiasm for its approval, we could speculate its use, in first line therapy of indolent B-NHL in regimens excluding chemotherapy. In the near future, the efficacy of the combination of new agents will be assessed in clinical studies. However, we should keep in mind the potential toxicities associated with some of these regimens, the quality of life of patients on long-term therapy (even when they are oral agents), and the social costs that might also be substantial. All these considerations will have to be assessed in order to further delineate the role of chemo-free strategies in the future daily management of patients with non Hodgkin's lymphoma.

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DIAGNOSTIC-PROGNOSTIC RELEVANCE OF MUTATIONS IN THE CHRONIC MYELOPROLIFERATIVE NEOPLASMS

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After decades of sluggish advancements, the Philadelphia-chromosome negative classic chronic myeloproliferative neoplasms (MPN), that include polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF), have witnessed a burst of knowledge as regards the underlying molecular abnormalities. In 2005 four reports described a

recurrent *JAK2*V6717F mutation[1] that is associated with >95% of PV and about 60% of ET and PMF; *JAK2* mutations located in exon 12 are found in an additional 2-3% of PV patients wild-type for the V617F allele, while 3% to 8% of ET and PMF patients have mutations in the gene thrombopoietin receptor, *MPL*. The diagnostic implications of these discoveries resulted in the revision of the diagnostic criteria of MPN by the WHO in 2008.[2] New mutations involving the gene calreticulin (CALR), a multicompartmental and multifunctional protein whose best known function is that of a Ca2+ binding chaperone in the endoplasmic reticulum lumen, were discovered last year in 60-80% of *JAK2* and *MPL* wild type patients with ET and PMF, pointing to about 10%-15% of ET and PMF[3] patients as being "triple negative" (see Table 1). The recurrent and specific association of *CALR* mutations with these two MPN warrants the incorporation of *CALR* mutations in a revised WHO classification.[4] Unlike *JAK2* and *MPL*, *CALR* mutations are heterogeneous, consisting of insertions or deletions in exon 9; a 52-bp deletion (Type I; 45-53% of all cases) or a 5-bp insertion (Type II; 32-41%) represent >80% of cases, with prevalence of Type I in MF. There may be subtle differences in Tyoe I *versus* Type II mutations. Remarkably, all mutations cause a frameshift resulting in a novel C-terminal peptide made up by a minimal 36 amino acid stretch. Several studies have shown that high allele burden of JAK2V617F mutation are associated with a polycythemia vera-like phenotype and are associated with an increased risk of thrombosis in ET, being formally incorporated in a clinical score (IPSET-thrombosis). Furthermore, accumulation of JAK2 mutated allele accompanies transformation of PV and ET to secondary myelofibrosis. On the other hand, *CALR* mutations may impart a more favorable phenotype in both ET and PMF. In the former, *CALR* mutations are associated with a lower risk of thrombosis,[5] while in PMF they define a category of patients with better survival compared with *JAK2*V617F mutated and particularly the triple negative patients. The favorable prognostic impact of *CALR* mutation is independent of clinical scores (IPSS, DIPSS-plus).[6] Patients with *CALR* mutation have a lower risk of developing anemia, thrombocytopenia, and marked leukocytosis compared with *JAK2*V617F mutated or triple negative.[7] In addition to mutations in the "phenotypic driver" genes mentioned above, other recurrent mutations have been found in genes involved in the epigenetic machinery (*TET2, ASXL1, DNMT3A, IDH1* and *2*) and the spliceosome (*SRSF2*, *S3BH1*, *U2AF*); these mutations are found in 5-25%of MPN patients,[1] with a prevalence of MF, and may have prognostic relevance. We reported that PMF patients harboring mutation in any-one of *EZH2, ASXL1, IDH1/2* and *SRSF2* constituted an IPSS- and DIPSS-plus score–independent "high molecular risk" (HMR) category associated with shorter OS and greater risk of leukemia compared with patients lacking above mutations ("low molecular risk"; LMR).[8] Identification of HMR patients may help in a more effective risk stratification particularly for stem cell transplantation (Table 2). More recently, we evaluated the additional prognostic value of the number of HMR mutated genes; the presence of 2 or more mutations predicted the worst survival (median 2.6 years *vs* 7.0 years for one mutation *vs* 12.3 years for no mutation) and were also associated with shortened leukemia-free survival.[9] In a large international study, we also demonstrated that the presence of *CALR* mutation maintained a favorable prognostic impact when combined with mutations in *ASXL1*, that resulted as the most significant of HMR genes. In fact, multivariable analysis identified a *CALR*wildtype/*ASXL1*mut mutational status as the most significant adverse risk category for survival in PMF.[6] Analysis of mutation status may have potential therapeutic relevance. Studies with the JAK1 and JAK2 inhibitor ruxolitinib in MF have shown similar clinical responses irrespective of *JAK2* as well as *CALR* mutation status. Furthermore, in a comprehensive mutation analysis that evaluated the impact of 14 MF-associated mutations on clinical outcomes in 166 patients included in COMFORT-II, we found that responses in splenomegaly and symptoms, as well as the risk of developing ruxolitinib-associated anemia and thrombocytopenia, occurred at similar frequencies across different mutation profiles.[10] Ruxolitinib improved survival independent of mutation profile in HMR patients compared with best available therapy.[10]

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Table 1. Phenotypic driver mutations in MPNs.

Gene/hotspot	PV	ET	PMF
JAK2 V617F	93-95%	53-64%	58-65%
JAK2 exon12	2-4%	0	0
MPL W515	0	3-5%	4-8%
CALR	0	16-33%	21-25%
"Triple negative"	3-5%	12-16%	9-11%

The values reported in this Table are derived from several studies, including the following: Vainchenker W, *et al. Blood.* 2011; 118:1723-35; Nangalia J, *et al. N Engl J Med*. 2013; 369:2391-405; Klampfl T *et al. N Engl J Med*. 2013; 369:2379 - 90; Rotunno G, *et al. Blood.* 2014; 123:1552-5; Rumi E, *et al. Blood.* 2014; 123:1544-51; Tefferi A, *et al. Am J Hematol.* 2014; epub; Tefferi A, *Leukemia.* 2014;epub; Gangat N, *et al. Eur J Haematol* 2014; epub; Chen CC, *et al. Ann Hematol* 2014; epub; Andrikovics H, *et al. Haematologica.* 2014; 99:1184 -1190; Qiao C, *et al. Haematologica.* 2014; epub.

Table 2. The impact of high-molecular risk mutations on the risk stratification of patients with PMF by the IPSS score.*

IPSS Risk Categories	*ASXL1* N. (%)	*EZH2* N. (%)	*SRSF2* N.(%)	*IDHs* N. (%)	N (%) Of HMR patients
LOW	24/162 (14.8%)	6/165 (3.6%)	7/151 (4.6%)	2/157 (1.3%)	**35/166 (21.1%)**
INT- 1	28/142 (19.7%)	6/143 (4.2%)	6/136 (4.4%)	6/142 (4.2%)	**34 /146 (23.4%)**
INT- 2	23/100 (23.0%)	4/99 (4.0%)	9/97 (9.3%)	2/96 (2.1%)	**31 /104 (29.8%)**
HIGH	27/65 (41.5%)	8/66 (12.1%)	16/63 (25.4%)	1/60 (1.7%)	**39/68 (57.3%)**

*Cervantes F *et al.*, Blood 113; 13: 2895-28901

LYMPH NODE MESENCHYMAL STROMAL CELLS REGULATE THE IMMUNE RESPONSE AGAINST LYMPHOMA

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Lymphoma microenvironment both in Hodgkin (H) and Non-Hodgkin (NH) lymphomas (L) is the site where neoplastic cells try to survive and escape from immune system-mediated recognition. It is conceivable that lymphoma cells can divert the immune response inducing the generation of myeloid derived suppressor cells and regulatory T cells which can limit the efficiency of effector anti-lymphoma lymphocytes. Lymph node (LN) fibroblast-like cells, defined as mesenchymal stromal cells (MSC) because they secrete extracellular matrix components and differentiate into connective tissue cells, can interfere with the immune recognition of lymphoma cells in several ways: a) producing immunoregulatory cytokines as TGF which affect the expression of the NKG2D activating receptor present on anti-lymphoma effector lymphocytes b) releasing decoy ligands of NKG2D such as MICA/B and ULBPs which impair the correct interaction between cytolytic T lymphocytes (CTLs) or innate cells, such as natural killer (NK) cells and gammadelta T cells, with lymphoma target cells. LNMSC can be identified in vivo as they express transglutaminase II and show a spindle-like shape. The amount of LNMSC is generally low: MSC can grow as characteristic fibroblastic colonies starting from about $1/10^5$-$1/10^6$ cells present in a LN specimen. LNMSC appear as small spindle cells when at confluence while when seeded at low concentration they show a large and flat shape. Generally, the expression of a series of surface and cytoplasmic markers can aid in defining LNMSC: indeed, these cells express at the cell surface CD73, CD105, CD90 and CD146 antigens. It is of note that LNMSC can also express some markers of embryonic stem cells as Sox-2 transcription factor, and molecules as poliovirus receptor (PVR) which are ligands of the DNAM1 activating receptor expressed on effector T and NK lymphocytes. Also, LNMSC bear the enzymes involved in the shedding of NKG2DL as Erp5, ADAM10 and ADAM17. LNMSC can differentiate into adipose or bone forming cells provided they are cultured in the appropriate medium. LNMSC can efficiently inhibit T cell proliferation to several mitogenic stimuli. This inhibition is maximal when co-cultures with lymphocytes are performed using a monolayer of LNMSC and lymphocytes are in contact with LNMSC. This inhibition is not present or it is less evident when lymphocytes are co-cultured with non-confluent LNMSC layers. This would suggest that different regulating effects can be found in the LN microenvironment depending on the presence of low or high amounts of LNMSC. It is to be determined whether LNMSC can give rise to regulatory T cells, as it happens for MSC isolated from bone marrow. In addition, pharmacological down-regulation of LNMSC-mediated immune-suppression of T cell responses and inhibition of LNMSC–mediated help to B cells can be a useful tool to favor immune cell response and limit B cell lymphoma growth. In this context, the aminobisphosphonates (N-BPs)-pulsed dendritic cells (DC) can trigger both activation and proliferation of gammadelta T cells and this expansion is thought to be dependent on the production of isopentenyl-pyrophosphate (IPP) which in turn stimulates gammadelta T cells. Recently, we found that the N-BPs zoledronic acid can be taken up by LNMSC isolated from HL and NHL; in turn, these MSC can stimulate, rather than inhibit, autologous gammadelta T cell proliferation. In addition, this effect in N-BPs-pulsed MSC is accompanied by the downregulation of TGFbeta production and the upregulation of IL15. This in turn leads to the generation of effector gammadelta T cells able to lyse tumor lymphoma cells more efficiently. Furthermore, this imbalance between IL15 and TGFbeta production leads to a strong increase of expression of Th1 cytokines as IFNgamma and TNFalpha. Since in HL and NHL lymph nodes, RT-PCR quantitative analysis revealed that cytokines as TGFbeta and IL10 typical of a Treg pattern are expressed while a Th1 or Th1-Th17 pattern is infrequent, N-BPs may be a tool for favoring the generation of an appropriate microenvironment for an optimal anti-lymphoma response. In conclusion, these findings strongly suggest that LNMSC may down-regulate the immune response in lymphoma microenvironment and that they may be targeted with appropriate drugs to trigger anti-lymphoma effector lymphocytes.

ROLE OF PROTEIN KINASE CK2 IN HAEMATOLOGIC MALIGNANCIES

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Abstract. Protein kinase CK2 is central for cell biology and is exploited by cancer cells due to its multifold activities on essential survival processes. The tetrameric assembly of two catalytic and two regulatory subunits composes this kinase in the cell. Non-oncogene addiction is the principal mechanism through which protein kinase CK2 sustains malignant cell phenotype. This kinase exerts its oncogenic function by enhancing pivotal growth-propelling signaling pathways. Recently, CK2 has emerged to be central in human B, T and myeloid cell derived hematopoietic malignancies. CK2 is over-expressed and over-active in acute lymphoblastic leukemia, multiple myeloma, chronic lymphocytic leukemia, mantle cell lymphoma, acute myeloid leukemia and myeloproliferative neoplasms.[1-5] In these tumors, CK2 controls the activation of the NF- B, JAK/STAT, PTEN/PI3K/AKT oncogenic signaling cascades, as well as the endoplasmic reticulum and proteotoxic stress response.[1,6]. Targeting CK2 in hematologic tumors could have the potential of boosting the cytotoxic effects of chemotherapeutic drugs as well as novel agents, like Hsp90, proteasome and tyrosine kinase inhibitors. Therefore, the availability of novel small ATP-competitive CK2 inhibitors could open new perspectives in the treatment of these tumors and will likely offer the opportunity to explore novel combination therapies with conventional drugs and last generation compound.

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MOLECULAR MECHANISMS OF RESISTANCE TO PI3K/AKT/mTOR TARGETED THERAPY IN LEUKEMIA

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The PI3K pathway has been found constitutively activated in leukemias, and is being robustly proposed as a therapeutic target for this disease.[(1-4)] By means of reverse phase protein arrays,[(5)] we examined the response of 80 samples of primary cells from AML patients to selective inhibitors of the PI3K/Akt/ mTOR axis. We found that more than 60% of the samples analyzed are characterized by high pathway phosphorylation.[(6)] Unexpectedly, however, we show also that while short-time treatment (2 and 4 hours) with the Akt or mTOR specific inhibitors Akti

1/2 and Torin1,[7-8] alone or in combination, results in strong Akt inactivation (Figure 1a), long-time treatment (20 hours) results in paradoxical phosphorylation of both Akt (at S473 and T308) and its direct substrates PRAS40, FOXO and GSK3 in 70% of the samples (Figure 1a). Thus we speculated that initial inhibition of Akt and/or mTOR activity stabilizes the Akt/mTOR downstream effectors FOXOs and IRS-1, which in turn potentiate signaling through up- regulation of the expression/phosphorylation of selected growth factor receptor tyrosine kinases (RTKs).[9] The analysis of the total content (Figure 1b), of the expression (Figure 1c) and of the phosphorylation (Figure 1d) of RTKs families such as IR/IGF-1R and PDGFR demonstrated that blunting Akt signaling indeed increases both expression and phosphorylation of the above receptors. Activation of RTKs in turn reactivates PI3K and downstream signaling, thus overruling the action of the drugs. The Combination Index values reported in Table 1, calculated according to Chou&Talalay,[10] further demonstrate that dual inhibition of Akt and RTKs displays strong synergistic cytotoxic effects in AML cells and down-modulates Akt signaling to a much greater extent than either drug alone, and should therefore be taken into account in AML clinical setting. Moreover, we are exploring the effectiveness of a different panel of drugs, namely GSK-690693 (8), GDC-0068 and PF-04691502, in the monocytic leukemia THP1 cell line. We show here that while the Akt inhibitors GSK-690693 and GDC-0068, at concentrations up to 10 micromolar for 72 hours, do not significantly affect cell viability (Figure 2a), the dual PI3K and mTOR inhibitor PF-04691502 is very effective in triggering apoptosis (Figure 2a) and does not induce compensatory signaling up to 72 hours after treatment (not shown). What's more, PF-04691502 has strong synergistic effect with Sunitinib, and is therefore effective at lower concentrations (Figure 2b). This is a very promising result which warrants further investigation in primary leukemia cells.

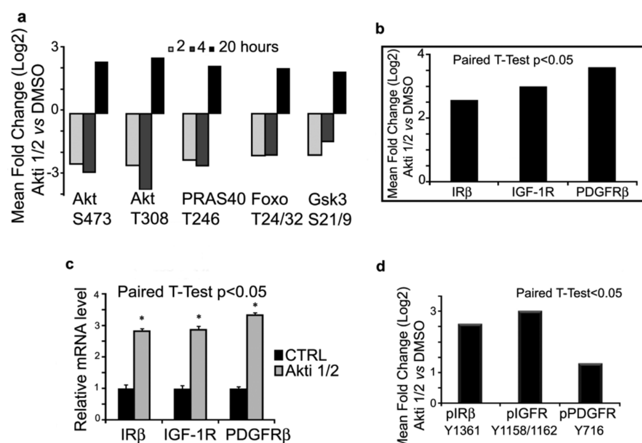

Figure 1. Long-time administration of Akt inhibitors to primary blasts leads to paradoxical Akt activation and RTKs up-regulation in all inhibitor-resistant primary AML cells. a). The inhibitor-resistant samples (n=35) were additioned with vehicle (DMSO) or with Akti 1/, followed by RPPA analysis of the indicated times and end points. b) The level of IR, IGF-1R and PDGFR protein was measured by ELISA in the 35 inhibitor-resistant samples treated as in (a). c) RT-PCR analysis of IGF1R, IRbeta and PDGFR mRNA expression in 7 samples from the inhibitor-resistant group treated as in (a). The data are represented as fold expression ± SEM, normalized with GAPDH gene expression. d) Phosphorylation, measured by RPPA, of IGF1R, IRbeta and PDGFR in the 35 inhibitor-resistant samples treated as in (a). Data in panels a, b, and d are represented as mean fold change (Log2) of the ratio treatment/DMSO (n=35).

Table 1. Combined treatment with PI3K pathway inhibitors and RTKs inhibitors displays synergistic effect. Cells expressing either IGF-1R (THP1), or PDGFR (MOLM13) were treated with Akt and RTK inhibitors as indicated for 72 hours, followed by MT assay.

		CI			
		ED50	ED75	ED90	ED95
THP1	Akti1/2+ Sunitinib	0.52	0.44	0.33	0.29
MOLM13	Akti1/2+ Linsitinib	0.48	0.36	0.32	0.21
MOLM13	Akti1/2+ Quizartinib	0.63	0.52	0.48	0.33

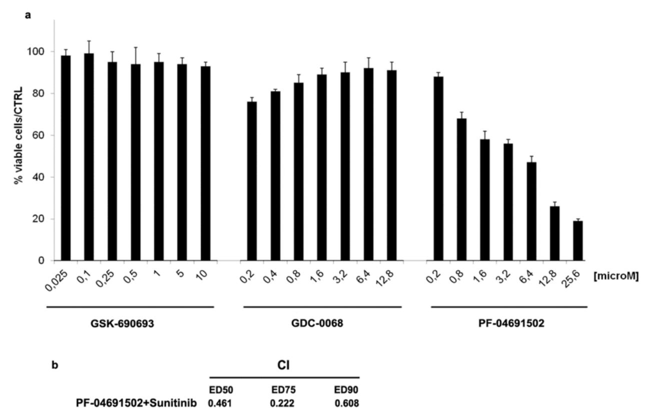

Figure 2. Inhibition of PI3K/mTOR dramatically decreases THP1 cells viability. Cells were additioned with vehicle (DMSO), GSK-690693, GDC-0068 or PF-04691502 at different doses as indicated, for 72 hours, either alone (a) or in combination with Sunitinib (b) followed by MTT analysis. PF-04691502 administration is cytotoxic, and diminishes cell viability both alone and in combination with the PDGFR family inhibitor Sunitinib. Combination Index (CI)<1 indicates synergism.

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HYPOXIA AND HYPOXIA-INDUCIBLE FACTOR (HIF)-1A SIGNAL PATHWAY ARE POTENTIAL TARGETS IN MULTIPLE MYELOMA

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Multiple myeloma (MM) is characterized by an extremely resistance to apoptosis and drug treatment.[1] MM cells induce alterations of the bone marrow (BM) microenvironment including increased angiogenesis and bone destruction that, in turn, support MM cell growth and drug resistance.[1] Hypoxia is a common feature of solid tumors and it is a critical trigger of the angiogenic switch by regulating the production of the pro-angiogenic molecules.[2] Tumor adaptation to hypoxia is mainly due to the HIF family molecules that regulate pro-angiogenic factors.[3] The HIFs are heterodimeric complexes composed of an inducible -subunit (HIF-1alpha, HIF-2alpha or HIF-3alpha) and a constitutively expressed -

subunit. Among the HIF family members, HIF-1 and HIF-2 are the best characterized. HIF-1alpha and HIF-2alpha have been shown to be the master regulators of oxygen homeostasis and they have pivotal roles in physiological angiogenesis and tumor progression.(3) In addition to neoangiogenesis regulation, a role of HIF-1alpha in osteoclast formation and development of osteolytic bone metastasis has been hypothesized in solid tumors such as breast cancer.(4) BM microenvironment has long been considered to be physiologically hypoxic. Recently, it has been demonstrated that the BM microenvironment in MM patients is hypoxic with an average oxygen tension of 52mmHg(5) and that MM cells overexpressed HIF-1alpha and HIF-2alpha.(5-6) Moreover, it has been shown that HIF-1alpha is up-regulated by MM cells in relationship with the presence of c-MYC translocation.(7) On the basis of these evidences, we hypothesize that HIF 1alpha is a key molecule involved in MM induced angiogenesis and osteolysis, and consequently its inhibition could have an impact on MM cell growth. Firstly, we explored the effect of persistent HIF-1 alpha inhibition by a lentivirus short hairpin RNA pool on MM cell growth, either *in vitro* or *in vivo*, and on the transcriptional and proangiogenic profiles of MM cells.(8) HIF-1alpha suppression did not have a significant impact on MM cell proliferation and survival *in vitro*. By gene expression profiling analysis, we identified several genes regulated by hypoxia and HIF-1alpha in MM cells. Particularly, we found that HIF-1alpha inhibition in MM cells down-regulates the pro-angiogenic genes *VEGF*, *IL8*, *IL10*, *CCL2*, *CCL5* and *MMP9*. Pro-osteoclastogenic genes were also inhibited, including *IL7* and *CCL3*. The effect of HIF-1a inhibition was then assessed *in vivo* in non-obese diabetic/severe combined immunodeficient mice, both in a subcutaneous and an intratibial MM model. HIF-1alpha inhibition caused a dramatic reduction in the weight and volume of the tumor burden in both mouse models. Moreover, a significant reduction of the number of vessels and was observed. Finally, in the intratibial experiments, HIF-1alpha inhibition significantly blocked bone destruction, as demonstrated by micro-QCT analysis. Secondly, we investigated whether HIF-1alpha suppression in MM cells could enhance the response to the current therapy overcoming drug resistance treatment with Lenalidomide (LEN) induced a significant higher inhibition of cell proliferation but not of cell viability in HMCL carrying HIF 1alpha suppression, whereas Bortezomib induced a similar rate of cell proliferation and cell death in MM cells. Consistently, we found that LEN increased p27 expression and down-regulated IRF4 expression in MM cells with HIF-1a suppressed. In addition, in MM mouse model, we found that LEN treatment induced a dramatic reduction of the weight and volume of the tumor burden in mice injected with JJN3 infected with anti-HIF-1 lentivirus vector as compared to JJN3 infected with the control vector pLKO.1. Overall these data indicate that HIF-1alpha suppression in MM cells significantly blocks MM-induced angiogenesis, reduces MM tumor burden and bone destruction *in vivo,* and increases the anti-MM effect of LEN, suggesting that HIF-1alpha and its related signal pathway are potential new therapeutic targets in MM.

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SIGNALING PATHWAYS AS THERAPEUTIC TARGET IN LYMPHOMAS

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Introduction. Genomic studies have led to a burst of disease-relevant molecular information in a variety of lymphoid tumors, including chronic lymphocytic leukemia (CLL), diffuse large B-cell lymphoma (DLBCL), mantle cell lymphoma (MCL), Waldenström macroglobulinemia (WM), hairy cell leukemia (HCL), and splenic marginal zone lymphoma (SMZL) (Figure 1). On the path toward personalized medicine of lymphoma patients, genomic aberrations driving tumor behavior represent an invaluable tool to improve therapeutic efficacy by targeted treatment. This review aims at covering the common signaling pathways that are molecularly deregulated in lymphomas, and how knowledge of the alteration of these pathways may contribute to disease management. Signaling pathways to witch lymphoma may be addicted include: *i)* B-cell receptor (BCR); *ii)* Toll-like receptor (TLR); *iii)* NOTCH; *iv)* nuclear factor- B (NF-B); and *v)* mitogen activated protein kinase (MAPK).[1]

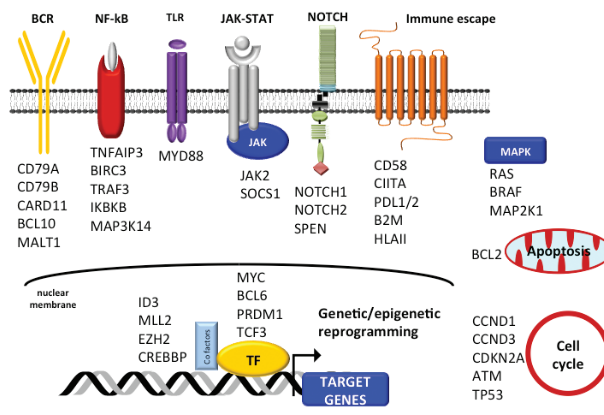

Figure 1. . Pathways that are molecularly deregulated in B-cell tumors.

B-cell receptor signaling. In B-cell tumors, mechanisms of BCR activation are quite diverse and range from chronic antigenic drive by microbial antigens to autostimulation of the BCR by self-antigens to activating mutations in intracellular components of the BCR pathway. Hepatitis C virus and Helicobacter pylori infection can lead to the development of marginal zone lymphomas. Successful treatment of the infection removes the inciting antigen and results in resolution of lymphoma. CLL has been recognized as a malignancy of auto-reactive B-cells and its clinical course is in part determined by the differential response of the malignant cells to BCR activation. In DLBCL (Table 1), recurrent mutations of genes belonging to the BCR pathway (*CD79A*, *CD79B*, *CARD11*) end in constitutively deregulated downstream BCR signals.[2] Several agents targeting key proximal kinases in the BCR pathway, including BTK, PI3K, and SYK have shown significant activity across a broad range of B-cell tumors, including MCL, CLL, DLBCL and WM. Upon the introduction of these agents in the clinic, bypass mutations that result in BCR inhibitor resistance and progressive disease are increasingly emerging, including mutations inhibiting drug binding to the target (*BTK*) and mutations activating the pathway downstream the target (*CARD11*, *PLCG2*, *BIRC3*).[3] *Toll-like receptor signaling.* Somatic mutations of *MYD88*, a key component of the TLR signaling machinery, occur in ~90% WM, ~30% DLBCL, ~10% SMZL and ~3% CLL (Table 1). Deregulated *MYD88* signaling promoted by mutations sustains tumor cell survival in WM and DLBCL, demonstrating that they are gain-of-function driver events in these lymphomas. Consistent with the notion that WM and DLBCL are addicted to *MYD88* signaling, genetic and/or pharmacologic knockdown of MYD88 or its downstream targets such as BTK, IRAK1/IRAK4, and TAK1 kills lymphoma

cells.[4] *NOTCH signaling.* The NOTCH pathway is molecularly deregulated in different mature B-cell tumors, including ~30-40% SMZL, ~10% CLL and ~5% MCL, by mutations affecting NOTCH receptors (*NOTCH1*, *NOTCH2*) or, less frequently, other genes involved in the downstream signaling (*FBXW7*, *SPEN*, *DTX1*, *MAML2*) (Table 1).[5,6] Pre-clinical studies have documented an addiction of CLL and MCL cells to NOTCH signaling for their survival, supporting the clinical investigation of NOTCH inhibitors in these tumors.[1] *Nuclear factor- B signaling.* One of the main downstream effector mechanisms of BCR, TLR and NOTCH signaling, which are recurrently mutated in B-cell tumors, is the activation of NF- B. NF- B signaling is also a direct target of genetic lesions in some B-cell tumors, including DLBCL and SMZL, which specifically rely on constitutive NF- B signaling (Table 1).

Table 1. Genetic lesions affecting signaling pathways in lymphoid tumors[a].

	DLBCL	WM	SMZL	MCL	CLL	HCL
CD79A mutation	20%	-	1%	-	-	-
CD79B mutation	20%	-	-	-	-	-
CARD11 mutation	10%	-	-	-	-	-
MYD88 mutation	30%	90%	10%	-	3%	-
NOTCH1 mutation	8%	-	5%	5%	11%	-
NOTCH2 mutation	8%	-	20%	-	-	-
SPEN mutation	-	-	5%	-	-	-
DTX1 mutation	-	-	2%	-	-	-
MAML2 mutation	-	-	2%	-	-	-
FBXW7 mutation	-	-	-	-	2%	-
TNFAIP3 mutation/deletion	30%	40%	7%	44%	-	-
IKBKB mutation	-	-	7%	-	-	-
BIRC3 mutation/deletion	-	-	10%	-	4%	-
TRAF3 mutation/deletion	-	-	5%	-	-	-
BRAF mutation	4%	-	-	-	-	100%

[a] DLBCL, diffuse large B-cell lymphoma; WM, Waldenström macroglobulinemia; SMZL; splenic marginal zone lymphoma; MCL, mantle cell lymphoma; CLL, chronic lymphocytic leukemia; HCL, hairy cell leukemia

In the activated B-cell type of DLBCL, the *TNFAIP3* gene, a negative regulator of NF- B signaling, is disrupted by mutations in ~30% of cases. *TNFAIP3* loss causes supra-physiological activation of NF- B signaling which, in turn, has oncogenic properties. In SMZL, NF- B activation is driven by genetic lesions targeting key regulators of both canonical (*TNFAIP3*, *IKBKB*) and non-canonical (*BIRC3*, *TRAF3*, *MAP3K14*) NF- B signaling.[7] *Mitogen activated protein kinase signaling.* Mutations of *BRAF*, one of the RAF family serine/threonine kinases in the MAPK signaling cascade, is the disease-defining genetic event of HCL. In HCL, *BRAF* V600E mutation leads to constitutive activation of the signaling, which appears to be crucial for HCL cell survival.[8] The finding of *BRAF* mutations as the main genetic driver of HCL supports the proposal of clinical evaluation of vemurafenib, a drug that specifically inhibits this mutant kinase. Beside pre-clinical evidences, the promising activity of this approach is also supported by anecdotal reports showing the efficacy of vemurafenib in treating patients with relapsed/refractory HCL.[9]

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LOSS OF *TET2* IN HEMATOPOIETIC CELLS LEADS TO DNA HYPERMETHYLATION OF ACTIVE ENHANCERS AND INDUCTION OF LEUKEMOGENESIS

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DNA methylation is tightly regulated throughout mammalian development and altered methylation patterns are a hallmark of human diseases, including hematologic malignancies. TET2 is a member of the TET family of proteins (TET1-3) that can convert 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), leading to site-specific DNA demethylation.[1] *TET2* is the only gene of the *TET* family that is mutated with high frequencies in patients suffering from a wide variety of hematopoietic diseases, including malignancies such as myelodysplastic syndrome (MDS), chronic myelomonocytic leukemia (CMML) and acute myeloid leukemia (AML), as well as B- and T-cell lymphomas.[2] In this study, we generated a physiologically relevant mouse model in which *Tet2* inactivation cooperates with AML1-ETO, the oncofusion protein emanating from the t(8;21)(q22;q22) translocation, to increase hematopoietic cell replating efficiency and proliferation *in vitro* and trigger AML development *in vivo*. Despite several reports, it is not clear how *TET2* mutations affect DNA methylation patterns in the genome and contribute to development of hematological disorders. Detailed analysis using reduced representation bisulfite sequencing revealed that loss of *Tet2* in hematopoietic cells leads to progressive hypermethylation of active enhancer elements and altered expression of genes implicated in tumorigenesis. In contrast, CpG island and promoter DNA methylation did not change in a *Tet2*-dependent manner. Furthermore, we demonstrate that enhancers are also hypermethylated in human AML patients with *TET2* mutations. Thus, we propose that *TET2* prevents leukemic transformation of hematopoietic cells by protecting enhancers from aberrant DNA methylation and that detailed analysis of enhancer methylation in TET2-mutated patients could potentially lead to the unraveling of the first molecular events that lead to initiation of the disease state.

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IN VITRO AND *IN VIVO* ACTIVITY OF CHIMERIC ANTIGEN RECEPTORS REDIRECTED AGAINST CD33 AND CD123 ANTIGENS FOR THE SPECIFIC TARGETING OF ACUTE MYELOID LEUKAEMIA

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The therapeutic outcome of acute myeloid leukaemia (AML) has improved over the last years, but this disease is still associated with high rate of relapse, challenging the scientific research in defining alternative treatment options. Adoptive immunotherapy with chimeric antigen receptor (CAR)-engineered T cells represents a fascinating biotechnological tool at the frontiers of modern cancer treatment.[1] CARs are artificial T-cell receptors constituted by an extracellular-antigen-binding domain derived from a monoclonal antibody and an intracellular-TCR triggering region. Thus, CAR engineered T cells exploit both the antigen binding properties of monoclonal antibodies and T-cell mediated effector functions (granzyme/perforin cytotoxicity and cytokine secretion).[2] Concerning AML, the CD33 and CD123 can be suitable target antigens to be targeted by CAR-redirected T cells, due to their overexpression profile on leukemic cells, in particular on leukemic stem cells (LSCs), the main responsible cellular compartment of the disease relapse.[3] Moreover, since CD33 is also expressed on normal haematopoietic stem/progenitors cells (HSPCs), resulting in a potential severe impairment of normal myelopoiesis, CD123 has emerged as an alternative attractive molecule based on its differential expression pattern, being widely overexpressed by AML population and less expressed on HSPCs.[4] Thus, the *in vitro* and *in vivo* efficacy and safety of this anti-AML CAR approach has been investigated genetically modifying Cytokine-Induced-Killers (CIK) cells to express CAR molecules specific for the CD33 or the CD123 antigen.Transduced cells were able to strongly kill $CD123^+$ cell lines*in vitro*, as well as primary AML blasts. Interestingly, secondary colonies experiments demonstrated that anti-CD123.CAR preserved *in vitro* HSPC*s* in

contrast to the anti-CD33.CAR, while keeping identical cytotoxicity profile towards AML. Furthermore, a limited killing of normal monocytes and CD123-low-expressing endothelial cells was measured, thus indicating a low toxicity profile of the anti-CD123.CAR.[5] Once injected into AML engrafted NSG mice, genetically modified T cells had a potent antitumour effect, both in a situation of low-AML engraftment and in mice with an established high AML burden. Secondary transplantation experiments on residual AML cells demonstrated that residual AML cells were still sensitive to CARs approach, leading to an almost complete eradication of the disease. Concerning the evaluation of the safety profile of such approach against normal haematopoietic precursors*in vivo*, mice injected with primary cord blood-derived CD34$^+$have been treated with both anti-AML CARs, showing anti-CD123.CAR to be better in sparing the normal hematopoietic compartment.[6] Taken together, our results indicate that CD123-specific CARs strongly enhance anti-AML CIK functions, while sparing HSPCs and normal low-expressing antigen cells, paving the way to develop novel immunotherapy approaches for AML treatment, particularly in the context of minimal residual disease in high-risk transplanted AML patients or for patients not eligible to receive high dose chemotherapy.

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ATYPICAL HEMOLYTIC UREMIC SYNDROME AND COMPLEMENT ACTIVATION

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Hemolytic uremic syndrome (HUS) is a rare disorder of microangiopathic hemolysis, thrombocytopenia and renal failure.[1] About 90% of childhood cases are caused by *E.coli* strains producing Shiga-like toxins (STEC-HUS) and have a good prognosis.[2] Shiga-like toxins (Stx1 and Stx2) are exotoxins that enter target cells after binding the glycolipid surface receptor globotriaosylceramide, abundantly expressed on the renal microvascular endothelium, and inhibit protein synthesis and cell death.[1,2] Sublethal amounts of toxin induce profound alterations in endothelial cells. Stx1 and Stx2 favour inflammation and induce loss of endothelial thromboresistance, leading to microvascular thrombosis, as documented by the formation of organized thrombi upon whole blood flowing on human microvascular endothelial cells pre-exposed to Stxs.[2] Less than 10% of cases are not caused by STEC. This atypical form (aHUS) has a worse outcome with a 10-15% mortality during the first clinical manifestation and up to 50% of cases progressing to end-stage renal disease (ESRD).[1] In the last 15 years, a clear link has been established between aHUS and defects in regulation of the alternative complement pathway,[1] which has paved the way for complement-tailored treatments. Approximately half of aHUS patients have mutations in *CFH, CFI* and *MCP*, encoding the complement regulatory proteins complement factor H, factor I and membrane-cofactor protein. In 3-5% of patients, hybrid genes deriving from uneven cross-over between *CFH* and *CFHR1* have been found. These genetic defects result in gene products with decreased complement regulatory activity on cell surfaces.[1] Inhibitory anti-CFH antibodies are reported in 5-10% of aHUS patients. They predominantly target the C-terminus of CFH, thereby impairing complement regulation on host cell surfaces. The development of CFH autoantibodies in aHUS has a genetic predisposition, being strongly associated with deletion of the CFHR1 gene.[1] About 3% of aHUS patients carry mutations in the gene encoding thrombomodulin (*THBD*), a membrane-bound anticoagulant protein that modulates complement. Gain-of-function mutations in key proteins of the alternative pathway, *C3* and complement factor B (*CFB*) have been reported in 10% and 1-2% of patients, respectively. Mutant proteins are resistant to inactivation by complement regulators.[1] The above defects lead to unrestricted complement activation, which eventually results in platelet, leukocyte and endothelial cell activation and microvascular thrombosis. Incomplete penetrance of aHUS has been reported in mutation carriers, indicating that additional genetic and/or environmental hits are necessary for disease manifestation. In an European survey including 795 patients from 4 cohorts, 3% of patients carried mutations in two or three complement genes.[3] Within families, the concomitant presence of *CFH* and *MCP* risk haplotypes increased aHUS penetrance in combined mutation carriers, further underlying the oligogenic feature of aHUS.[3] About 40% of patients with aHUS do not carry complement gene mutations nor anti-CFH antibodies. Complement activation in aHUS is restricted to cell surface level, while activation in fluid phase is normally controlled. As a consequence measurement of complement activation parameters in serum and plasma, like C3 and sC5b-9 levels, often fails to find out abnormal values. At variance, specific assay of complement activation on endothelium consistently evidence abnormal complement deposits on cell surface not only in patients with complement gene mutaitons but also in patients without identified genetic abnormalities. Short and long-term outcomes and the risk of recurrence after transplantation vary [4,5] according to the underlying complement abnormality. All these studies represented a strong rationale for use of the anti-C5 humanized monoclonal antibody Eculizumab in aHUS. This drug**,** by blockade of C5 cleavage**,** protected from microvascular thrombosis and radically improved the outcome of aHUS.[6] Recently, homozygous or compound heterozygous mutations in the gene encoding for the diacylglycerol kinase (DGKE) co-segregated with aHUS in nine unrelated kindreds.[7] Mutation carriers presented with aHUS before 1 year of age, had persistent hypertension, haematuria and proteinuria and developed chronic kidney disease with age. DGKE is expressed in endothelium, platelets and podocytes. It is apparently unrelated to the complement cascade. However a more recent paper[8] identified a novel DGKE mutation in a consanguineous family with patients affected by aHUS characterized by serum complement activation. Aggressive plasma infusion therapy controlled systemic symptoms and prevented renal failure, suggesting that this treatment can significantly affect this disease. Mechanistic studies of DGKE and aHUS are, essential to the design of therapeutic strategies in patients with DGKE mutations.

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ANTISENSE-BASED THERAPY OF COAGULATION FACTOR V DEFICIENCY

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Introduction. Antisense technology is emerging as a promising tool to correct splicing defects.[1] Antisense molecules, including synthetic DNA analogues and engineered small nuclear RNAs (snRNAs), are designed to anneal to a specific pre-mRNA and to hide incorrect/undesired splicing signals, in order to direct splicing in the correct/desired way. This form of molecular therapy holds promise as a molecular therapy for several genetic diseases caused by splicing mutations. In particular, it may be a valid alternative to fresh frozen plasma in the management of coagulation factor V (FV) deficiency, a rare bleeding disorder for which no specific factor concentrate or recombinant preparation is available.[2] Recently, we investigated a 35-year-old male patient with undetectable FV and multiple life-threatening bleeding episodes (including two spontaneous intracranial haemorrhages). This patient was homozygous for a deep-intronic splicing mutation (*F5* IVS8+268A>G) which activates a cryptic donor splice site and causes the inclusion of a pseudo-exon with an in-frame stop codon in the mature *F5* mRNA.[3] *Aim.* To design antisense molecules targeting the aberrant splice site and to test their efficacy in an *in vitro* minigene model and *ex vivo* on patient-derived megakaryocytes. *Methods.* COS-1 (kidney) and HepG2 (liver) cells transfected with a *F5* minigene construct containing the IVS8+268A>G mutation were treated with an antisense morpholino oligonucleotide (MO, 0-10 micromolar) or with a construct (0-2 microgram) expressing antisense U7snRNA. After 48 hours, mRNA was analysed by real-time qPCR and gel electrophoresis. *Ex vivo* experiments were approved by the Ethics Committee of Padua Academic Hospital and conducted with the patient's informed consent. Patient's megakaryocytes were obtained by *ex vivo* differentiation of circulating haematopoietic progenitors and FV expression was visualised by immunofluorescence staining. *Results.* Expression of the mutant *F5* minigene in COS-1 and HepG2 cells produced aberrant mRNA and normal mRNA in the proportion of ~10:1. Treatment with mutation-specific antisense MO and U7snRNA dose-dependently increased the relative amount of correctly spliced mRNA up to ~30-fold and ~100-fold, respectively, whereas control MO and U7snRNA with irrelevant sequences had no effect.[4] Patient's megakaryocytes did not express FV, but became positive for FV after treatment with mutation-specific antisense MO or U7snRNA.[4] Figure 1.

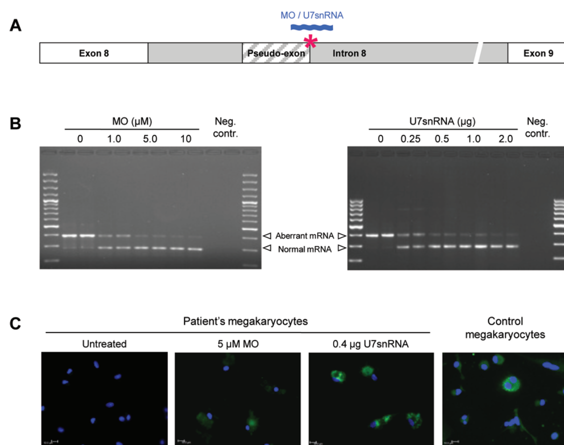

Figure 1. Splicing correction by antisense molecules. A) Correction strategy. The red asterisk marks the position of the IVS8 +268A>G mutation and the blue ribbon represents the antisense molecules used to hide the incorrect donor splice site. B) *In vitro* correction model. COS-1 cells transfected with the mutant *F5* minigene were treated with increasing concentrations of mutation-specific MO (0-10 micromolar) or U7snRNA (0-2 microgram). After 48 hours, mRNA was isolated and analysed by agarose gel electrophoresis. C) *Ex vivo* correction model. Patient's and control megakaryocytes were obtained by *ex vivo* differentiation of circulating haematopoietic progenitors. FV protein expression in untreated cells and in cells treated with mutation-specific MO (5 micromolar) or U7snRNA (0.4 microgram) was visualised by immunofluorescence staining (green colour).

Conclusions. Mutation-specific antisense molecules could correct the patient's *F5* splicing defect *in vitro* and restore FV synthesis in the patient's megakaryocytes *ex vivo*. These findings provide proof-of-principle for the efficacy of antisense-based RNA therapy in severe FV deficiency.

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SOMATIC MUTATIONS OF CALR IN MYELOPROLIFERATIVE NEOPLASMS

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Our understanding of the genetic basis of myeloproliferative neoplasms (MPNs) began in 2005, when the JAK2 V617F mutation was identified in polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). JAK2 exon 12 and MPL exon 10 mutations were then detected in subsets of patients. Recently, somatic mutations in the gene CALR, encoding calreticulin, have been found in 20-25% of patients with ET or PMF and are mutually exclusive with lesions in both JAK2 and MPL, indicating CALR as the second most frequently mutated gene in MPNs. All CALR mutations are indels clustering in the last exon of the gene. Thus far, more than 50 different types of mutations in CALR have been detected, but a 52-bp deletion (type 1 mutation) and a 5-bp insertion (type 2) are the most frequent types, overall found in more than 80% of all patients with mutant CALR. Interestingly, type 1 mutation is significantly more frequent in PMF than in ET, suggesting a particularly active role of the 52 bp-deletion in determining marrow fibrosis and extramedullary hematopoiesis. All these mutations cause a frameshift to the same alternative reading frame and generate a novel C-terminal peptide in the mutant calreticulin, in which the negatively charged amino acids of the calcium-binding domain are replaced by neutral and positively charged ones. In addition, the endoplasmic reticulum retention motif KDEL at the end is lost in the mutant variants. This suggests that both impaired calcium-binding activity and cellular dislocation may play a role in the abnormal proliferation of cells expressing a mutant calreticulin. Outside ET and PMF, CALR mutations are found in patients with refractory anemia with ring sideroblasts associated with marked thrombocytosis, suggesting a strict relationship between mutant CALR and thrombocytosis phenotype within myeloid neoplasms, and consequently a role of CALR mutations in affecting the biology of megakaryocytes. Studies on the impact of the 3 founding driver mutations (JAK2, MPL, and CALR) on clinical phenotype, risk of evolution into acute myeloid leukemia (AML) and overall survival (OS) showed that in ET, CALR mutations correlated with younger age, higher platelet count, and lower thrombotic risk; in PMF, they correlated with younger age, lower leukocyte count, higher platelet count, and lower IPSS and DIPSS risk distribution. Survival was favorably affected by CALR mutations in PMF but not in ET, while nonmutated JAK2, MPL and CALR PMF patients have very poor survival with a particularly high risk of leukemic evolution, even after adjustment for age. Thus, accounting for JAK2, CALR, and MPL mutation status is of fundamental diagnostic and prognostic relevance in myeloid neoplasms, especially when bone marrow fibrosis is present. It also provides a new powerful tool for understanding the molecular basis of myeloproliferative neoplasms, indicating a role of megakaryocytes in the pathophysiology of MPNs. Patterns of clonal evolution and phenotypic switch in these disorders are also suggested.

BEST ABSTRACTS

BEST01

NON HOMOLOGOUS END JOINING AS A NEW PROGNOSTIC FACTOR AND POTENTIAL TARGET IN MULTIPLE MYELOMA

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Introduction. Genomic instability is a hallmark of several types of solid and hematologic malignancies, including multiple myeloma (MM) in which structural and numerical chromosomal abnormalities are common features. However, the underlying molecular basis of MM genomic instability is still largely unknown. To this aim, we investigated the activity of non-homologous end joining (NHEJ), which represents the most important mechanism of double-strand breaks (DSBs) repair, in MM cells.We first assessed the NHEJ activity in MM cells and normal cells. To this aim, we developed and validated a dual gene plasmid-based assay utilizing Luciferase (LUC) as a test gene which measures end joining, and Alkaline Phosphatase (SEAP) as a reporter gene to control for transfection efficiency, in either intact cells (*in vivo* assay) or in cell free extracts (*in vitro* assay). The first one is a chemiluminescent assay which allows for direct measurement of LUC and SEAP in the supernatant of the cells 24h after electroporation with the plasmid, while the cell free extract method is a customized TaqMan® approach based on a quantitative evaluation of the plasmid rejoining. Both assays revealed a significant increase in NHEJ in all 6 MM cell lines tested compared to normal peripheral blood mononuclear cells (PBMC) and bone marrow stromal cells (BMSC). We further confirmed the hyper-activation of the NEHJ pathway by analyzing the binding activity of ku86, a key NHEJ-related protein involved in the recognition of the broken DNA ends and in the initiation of the DSBs repair process. Six out of 9 MM cell lines showed a significant increase in ku86-binding activity respect to normal cells. We also found an augmented phosphorylation at Ser 2056 of DNA-PK, a ku86-partner which plays a key role in NHEJ. Subsequently, in order to evaluate the role of NHEJ in MM genomic instability, we assessed the EJ activity of 4 MM cell lines after DNA-PK and/or PARP inhibition as well as ku86 sh-RNA knock-down. Interestingly, preliminary *in vitro* results suggested a potential involvement of the more errore-prone Alternative-NHEJ (A-NHEJ) pathway in the EJ activity of some MM cell lines. Next, we evaluated the NHEJ activity in 15 patient samples using the cell free assay. Interestingly, level of NHEJ activity divided patients into two different groups: one with an NHEJ activity similar to normal cells and the other to the MM cell lines. Moreover, using the French (IFM) and the Arkansas (GSE2658) dataset, we demonstrate a significant association between NHEJ pathway-related gene expression and overall survival by the Globaltest analysis. Finally, using a NHEJ inhibitor we demonstrated an inverse correlation between recombination activity and drug sensitivity. *Conclusions.* Our data demonstrate an aberrant activation of NHEJ in MM highlighting its role in patient outcome and suggesting this pathway as an important new prognostic marker as well as a potential target in MM.

BEST02

HIGH-THROUGHPUT SEQUENCING FOR THE IDENTIFICATION OF BRAF MUTATIONS IN MULTIPLE MYELOMA

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Introduction. BRAF is a serine-threonine kinase implicated in the pathogenesis of multiple myeloma (MM). It represents a drug target in malignant melanoma and, recently, BRAF inhibition has been reported as successful treatment also in a BRAF-mutated MM patient. *Methods.* To analyze BRAF mutation in untreated MM, we investigated by next generation sequencing (NGS) a retrospective cohort of 122 cases at onset, seven of whom were also tested at relapse after first line therapy. Moreover, we examined 11 patients with secondary plasma cell (PC) leukemia (sPCL). Deep sequencing of exons 11 and 15 was performed by Roche 454 pyrosequencing on the Genome Sequencer Junior instrument. *Results.* NGS analysis of BRAF mutation hot-spots in our cohort revealed the presence of 9 coding non-synonymous variants. Mutant allele frequency ranged from 1.27% to 51.88% of total reads (median depth of coverage 252x, range: 100-962). All the identified mutations were missense single nucleotide variations (SNVs). After excluding variants with an allele frequency lower than 2% (which will be subjected to independent 454 sequencing runs to verify their occurrence), six SNVs were found, three of which have been already described by others in MM patients (G469A, V600E, D594G): the remaining three mutations occurred at positions known to be targeted by mutational events, but caused an amino acid substitution to date unreported in MM (D594N, G596R, K601T). Overall, the prevalence of BRAF mutation in our cohort was 9.8% (12/122) in MM, and 18.2% (2/11) in sPCL. The great majority of identified mutations (83%) affected exon 15. In none of the seven MM patients tested also at relapse (all of which were wild-type at onset) BRAF mutations occurred during disease course. We identified four recurrent mutations: in particular, the V600E variant affected five patients; D594N was found in three cases; and D594G and G469A recurred each in two samples. In no case we observed the presence of more than one variant. BRAF mutations were not associated with the occurrence of IGH translocations nor with del(13). *Conclusions.* Our data confirm recent findings identifying BRAF as a recurrently mutated gene in MM; BRAF mutations, as well as KRAS and NRAS alterations, frequently occurring in MM, contribute to the activation of MAP kinase signaling, and thus connote MAPK pathway as a promising therapeutic target in myeloma.

BEST03

GENE THERAPY OF ACUTE LEUKEMIAS WITH CYTOKINE INDUCED KILLER (CIK) CELLS ENGINEERED TO EXPRESS CHIMERIC ANTIGEN RECEPTORS (CARS) BY SLEEPING BEAUTY

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Introduction. T cell engineering with CARs has been recently proved to be effective in redirecting effector activity towards leukemic blasts. Since the profile of efficacy, safety and feasibility of cell manufacturing and gene therapy by viral vectors still remain major concerns, we explored here the use of Sleeping Beauty (SB) Transposon-mediated gene transfer in CIK cells for targeting Acute Leukemias. *Methods.* With an optimized clinical-grade stimulation protocol, we genetically modified CIK cells to express two distinct CARs specific for myelogenous leukemia (AML) CD123+ or acute lymphoblastic leukemia (ALL) CD19+ blasts. *Results.* The nucleofection minimally affected the phenotype of CIK cells, and the optimized protocol was effective in inducing

T-cell expansion, with a fold increase sufficient to be translated into clinical protocols. Modified CIK cells displayed stable expression of CD123.CAR or CD19.CAR with a frequency of 51.4%±2.9 (n=13) and 48.8%±6.8 (n=7), respectively, and exerted efficient lysis of leukemic primary blasts. Interestingly, CAR triggering by the antigen expressed by leukemic cells promoted specific cytokine secretion and proliferation that was restricted to the modified fraction of CIK cells. The loss of the expression of transposase during the differentiation was assessed to assure the genome stability of the cellular product by absolute quantification through RT-PCR. Finally, insertion-site analysis by LAM-PCR confirmed that the integrations were distributed throughout the genome with frequencies comparable, indicating polyclonality. Conclusions. SB system together with an optimized method of differentiation efficiently expand CD123.CAR+ and CD19.CAR+ CIK cells, redirect their activity towards AML and ALL cells, and retain a safe pattern of integrations in the genome. An easy clinical-grade adoptive cell therapy platform based on an innovative non viral method of gene transfer will be fundamental to improve the range of applications of immunotherapy to control relapse in leukemic patients.

BEST04

THE KRÜPPEL-LIKE FACTOR 2 (KLF2) TRANSCRIPTION FACTOR IS RECURRENTLY MUTATED IN SPLENIC MARGINAL ZONE LYMPHOMA

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Introduction. Splenic marginal zone lymphoma (SMZL) is characterized by mutations of genes involved in physiological differentiation of marginal zone (MZ) B-cells. To identify additional molecular mechanisms responsible of SMZL pathogenesis, we investigated candidate genes that are well established regulators of MZ B-cells differentiation, but did not emerge from previous genomic studies. *Methods.* Seventeen genes were investigated by mutational analysis. Copy number abnormalities were assessed by SNP array and/or FISH. Expression was assessed by biochemical assays and immunohistochemistry. Wild type and mutants were conditionally expressed in HEK-293T, Jurkat, OCI-Ly8 and VL51 cells. *Results.* The zinc finger transcription factor KLF2 was somatically mutated in 20% (19/96) SMZL, thus representing one of the most frequently altered genes in this lymphoma, along with NOTCH2 (20%) and MLL2 (15%). Beside mutations, KLF2 was deleted in 11% (11/96) SMZL, including one focal loss encompassing the sole KLF2 locus. By combining mutations and deletions, 30% (30/96) SMZL harbored KLF2 lesions. By extending the analysis to 547 mature B-cell tumors, in addition to SMZL, KLF2 was also mutated in 16% (4/24) hairy cell leukemia, 15% (12/77) non-germinal center (GC) diffuse large B-cell lymphoma (DLBCL), 9% (5/56) nodal marginal zone lymphoma and 8% (5/61) extranodal marginal zone lymphoma. Conversely, KLF2 mutations were rare or absent in GCB-DLBCL, follicular lymphoma, Burkitt lymphoma, mantle cell lymphoma, chronic lymphocytic leukemia, and multiple myeloma. Most (62%) of KLF2 mutations disrupted its nuclear localization signal (NLS) and/or affected codons required for the interaction between KLF2 and DNA. In normal MZ B-cells of the spleen and in wild type tumors, KLF2 was preferentially expressed in the nucleus while it was aberrantly displaced from the nucleus in cell lines and primary tumor cells harboring NLS mutations. Consistently, transfection of HEK-293T and OCI-Ly8 cells showed a predominantly nuclear localization of the wild type KLF2, while NLS mutants were dislocated into the cytoplasm. The CDKN1A promoter, a known KLF2 direct target, was strongly induced by wild type KLF2 in luciferase assays. In contrast, KLF2 mutants failed to efficiently upregulate the reporter. Trunsduction of wild-type KLF2 upregulated the expression of the endogenous CDKN1A mRNA and protein, activated the apoptotic cascade, and enhanced cell death in lymphoid cell lines. Conversely, transduction of transactivation defective mutants were unable to induce the expression of the endogenous CDKN1A and did not show apoptotic activation. *Conclusions.* These data indicate that: i) KLF2 is a putative novel tumor-suppressor gene recurrently disrupted in 30% SMZL and, to a lesser extent, in other B-cell tumors; and ii) KLF2 mutations promote cell survival by causing the cytoplasmic delocalization of KLF2 and the impairment of its transcriptional function.

BEST05

BIOLOGICAL AND THERAPEUTIC RELEVANCE OF THE BRAF-MEK-ERK PATHWAY IN HAIRY CELL LEUKEMIA

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Introduction. Hairy cell leukemia (HCL) is a mature B-cell malignancy with unique clinico-biological features. The BRAF-V600E activating kinase mutation defines HCL among other B-cell lymphomas, including the HCL-like mimics splenic marginal zone lymphoma and HCL-v. The BRAF-MEK-ERK pathway thus emerges as an ideal candidate to illuminate the peculiar biology of HCL and as a therapeutic target to be attacked with clinically available BRAF and MEK inhibitors. However, a comprehensive mechanistic dissection of the effects of these inhibitors in primary HCL cells has not been conducted so far. To analyze *in vitro* the effects of BRAF and MEK inhibition in HCL, using patients' hairy cells (since the putative "HCL" cell lines lack BRAF-V600E, questioning their true HCL origin). *Methods.* Blood leukemic cells, purified from 23 HCL and 10 HCL-like patients using CD19-MACS, were treated *in vitro* with a BRAF inhibitor (Vemurafenib, PLX4720 or Dabrafenib) or the MEK inhibitor Trametinib, at concentrations up to 1 μM, for different time periods (up to 96 hours), and monitored for i) MEK/ERK phosphorylation by Western blotting; ii) downstream transcriptional changes by genome-wide expression profiling (GEP); iii) surface morphology changes by confocal microscopy following phalloidin/ANXA5 staining to highlight the F-actin-rich hairy projections in still living cells; iv) viability (by MTT or WST metabolic assays) and apoptosis (by AnnexinV staining). *Results.* Treatment with any BRAF inhibitors resulted in dose-dependent, sustained MEK and ERK dephosphorylation in all HCL cases, as opposed to vehicle-treated HCL cells and to inhibitor-treated HCL-like cells. Also Trametinib strongly dephosphorylated ERK in HCL cells. Interestingly, GEP after 48h and 72h of BRAF inhibition showed: i) silencing of the BRAF-MEK-ERK pathway transcriptional output; ii) loss of the HCL-specific GEP signature previously described by us (J Exp Med 2004;199:59); and iii) downregulation of the HCL immunophenotypical markers CD25, TRAP and cyclin-D1. Loss of surface CD25 and of nuclear cyclin-D1 was validated *in vivo* in HCL patients being treated with Vemurafenib in our HCL-PG1 phase-2 clinical trial. These *in vitro* biochemical and transcriptional events were followed by a consistent: i) loss of the hairy projections in still viable (ANXA5-negative) leukemic cells; ii) reduction of metabolic viability (up to 51.7% relative to the drug vehichle); and iii) decrease of living, non-apoptotic (ANXA5/PI-negative) cells (up to 84.4% relative reduction), all of this occurring specifically in leukemic cells of the vast majority of HCL patients as opposed to none of the HCL-like patients (Figure 1). *Conclusions.* This is the first thorough biological and pre-clinical dissection of: i) the prominent role of BRAF-V600E in shaping the specific transcriptional signature, morphology and immunophenotype of HCL; and ii) the significant anti-leukemic activity of BRAF or MEK inhibition in HCL.

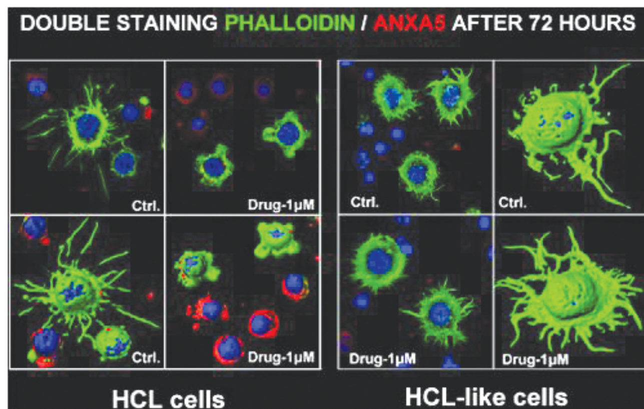

Figure 1.

ORAL COMMUNICATIONS

Leukemias and Myelodysplasias I

C001

PRECLINICAL EVALUATION OF THE PI3K INHIBITOR, BKM120, IN ACUTE MYELOID LEUKEMIA

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Introduction. The constitutive activation of PI3K/Akt/mTOR pathway is a common feature of acute myeloid leukemia (AML) and has been linked to disease progression, chemoresistance and poor prognosis. Therefore, the inhibition of this pathway represents an additional potential strategy for amelioration of AML therapy. BKM120 (Novartis) is a highly selective pan-class I PI3K inhibitor which has been evaluated with encouraging results on various solid tumors and hematological malignancies. Here we investigated the *in vitro* activity of BKM120 on AML. *Methods.* BKM120 was tested on AML cell lines and primary AML samples at concentrations ranging from 0.5 to 5 µM. Cell cycle and apoptosis were analyzed by Acridine-Orange and AnnexinV (AnnV)/PI staining. Intracellular signaling modulations and oxygen consumption rate (OCR) were evaluated by Western Blot analysis and the XF24 Flux Analyzer. *Results.* AML cell lines (U937, NB4, OCI-AML2, OCI-AML3, MOLM-13, THP-1, HL-60/MX2, HL-60 and KG-1) were characterized by constitutive activation of PI3K/Akt/mTOR axis. BKM120 exposure induced a dose-dependent dephosphorylation of p-Akt(Ser473) and of p-GSKα/β(Ser21/9), suggesting a functional blockade of the PI3K pathway. In addiction, BKM120 affected both mTORC1 and mTORC2 activity downregulating p-mTOR(Ser2448), p-p70S6K(Ser371) and p-4EBP1(Thr37/46) (mTORC1 downstream targets) and p-mTOR(Ser2481) (mTORC2). Selective inhibition of the PI3K/Akt/mTOR pathway correlated with a marked reduction of cell growth (IC50s at 72h ranging from 0.7 for U937 to 1.2µM for KG-1) and with a significant ($p<0.005$) dose-and time-dependent apoptosis induction in all tested cell lines. BKM120 cytotoxic effect was confirmed on 14 primary AML samples: a mean increase of AnnV positive cells from 18.7%±0.1 (DMSO) to 28.6%±0.2, 30.7%±0.2, 41.0%±0.2 ($p<0.001$) and 45.2%±0.2 ($p<0.001$) was obtained at 144h with 0.5, 1, 2 and 5µM, respectively. Dephosphorylation of p-Akt(Ser473), achieved at 24h, supported the target inhibition of BKM120 on primary AML samples. Moreover, BKM120 failed to induce apoptosis on normal and activated PBMCs isolated from 5 healthy donors (8.0% and 4.4% apoptosis net increase at 5 µM, respectively). In addition, we documented that BKM120 affected the OCR of AML in a dose-dependent manner inducing a marked reduction of basal and maximal respiration (72.9% and 59.9% with 2µM, respectively) and of the ATP production (97.0%) on the U937 cell line, compared to control. Similar effects were observed on primary AML samples. *Conclusions.* BKM120 significantly impaired proliferation, induced apoptosis and affected the OCR of AML cell lines and primary samples by the selective inhibition of PI3K/Akt/mTOR signaling, suggesting its potential therapeutic role on AML.

C002

CD123 (IL-3RA) IS OVEREXPRESSED ON CD34+CD38- PUTATIVE LEUKEMIC STEM CELL IN ACUTE MYELOID LEUKEMIA (AML) CARRYING NUCLEOPHOSMIN (NPM1) GENE MUTATION

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Introduction. CD123, the alfa subunit of the IL-3 receptor, is a transmembrane protein involved in cell survival and proliferation. It has been identified as a potential immunotherapeutic target because it is overexpressed in AML compared to normal hematopoietic stem cells and it

was also reported to be highly expressed on CD34+CD38- putative AML stem cells. Preclinical studies either with CD123xCD3 bispecific antibodies or CD123 CAR-engineered T cells have already validated CD123 as immunotherapeutic target in AML and clinical trials are consequently expected in the next future. NPM1 mutation is the most common genetic lesion in AML accounting for about 30% of cases. Previous cytometrical and immunohistochemical studies have shown that in NPM1-mutated as well as in FLT3-ITD AMLs, CD123 is frequently overexpressed. However, a comprehensive and quantitative evaluation of CD123 expression in AML bulk cells and on CD34+CD38- cells has not been reported. *Methods.* CD123 expression levels were evaluated by flow cytometry in fresh samples from 138 consecutive adult AML patients at diagnosis, using an antibody combination including CD34-FITC/CD123-PE/CD45-PerCP-Cy5.5/CD38-APC, reporting both percentage of CD123 positive cells (PPC) and CD123 median fluorescence intensity (MedFI) in both bulk and CD34+CD38- cell population. Blasts were gated using FSC/SSC and CD45/SSC dot-plots. *Results.* In our patient cohort 64/138 (46%) carried NPM1 gene mutation; 31/99 (31%) were FLT3-ITD mutated; only two patients displayed D835 mutation (1,4%) and were excluded from the analysis. According to karyotype, CD123 was more expressed on bulk cells (n=96) but not on CD34+CD38- cells (n=74) in AML with normal karyotype compared to AML with one or more karyotype abnormalities (mean values of MedFI respectively 37.8 *vs* 19.3, p<0.01 and 59.6 *vs* 28.3, p=0.23, Mann-Whitney). NPM1-mutated (NPMmut) AML showed brighter CD123 expression on both bulk cells (n=138) and CD34+CD38- cells (n=109) compared to NPM1 wild type (NPMwt) AML (mean values of MedFI 36 *vs* 18.5, p<0.01, and 73.3 *vs* 24.7, p<0.01, Mann-Whitney). According to FLT3 gene status, FLT3-ITD AML were associated with higher CD123 expression on both bulk cells (n=99) and CD34+CD38- cells (n=75) when compared to the FLT3 wild-type (FLT3wt) counterparts (mean values of MedFI respectively 50 *vs* 25, p<0.01, and 72.5 *vs* 38.3, p<0.01, Mann-Whitney). As shown in Figure 1 the highest expression was reported in NPMmut/FLT3-ITD genotype AML in both bulk and CD34+CD38- leukemic cell populations (mean value of MedFI 53.6 and 93.4, respectively). *Conclusions.* NPM1 and FLT3-ITD mutations correlate with higher CD123 expression levels in AML. Although CD123 was globally positive in most cases of AML in our series, it was markedly expressed in AML carrying mutations of both NPM1 and FLT3 either on leukemic bulk cells as well as on CD34+CD38- putative LSCs, making this AML subtype a suitable candidate for CD123-targeted immunotherapy.

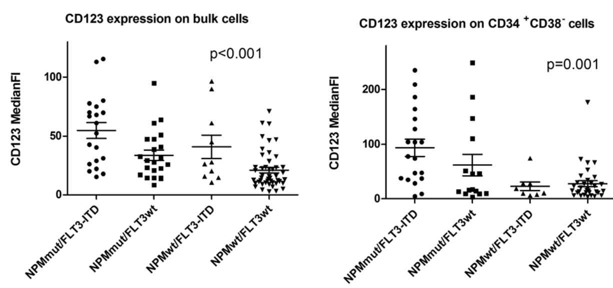

Figure 1.

C003

PAX5 FUSION GENES DRIVE STAT5 SIGNALING PATHWAY THROUGH LCK OVER EXPRESSION

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PAX5 is a transcription factor acting both as an activator and a repressor, known to be very frequently altered in B Cell Precursor Leukemia (BCP-ALL). PAX5 aberrations include point mutations, deletions, amplifications and translocations with several partner genes. All the originated fusion genes retain the PAX5 DNA binding domain but acquire the regulatory regions of the partner genes, showing a dominant negative mechanism on endogenous PAX5. However, the function of PAX5 fusion proteins and their common mechanism of leukemogenesis are poorly understood. We have established an *in vitro* model in which wt pre-BI cells are transduced with the most recurrent PAX5 fusion gene PAX5/ETV6. Gene expression profiling of transduced PAX5/ETV6 pre-BI cells showed a significantly altered transcription profile compared to the empty vector control. Interestingly, pathway analyses revealed that many genes implicated in the pre-B-cell receptor (pre-BCR) assembly and signaling were repressed by PAX5/ETV6. In addition, the downstream signaling of the BCR was non-functional through a block in IgM heavy chain rearrangement. Aim of the current study has been to elucidate the signaling pathway activated in PAX5/ETV6 transduced pre-BI cells and in primary patients carrying different PAX5 translocations in order to overcome the absence of a functional BCR expression. Interestingly, among the top-ranking over-expressed genes in PAX5/ETV6 pre-BI cells we found the PAX5 repressed target gene Lck, a member of the Src family kinase. Indeed, we demonstrated Lck up-regulation in PAX5/ETV6 positive pre-BI cells and in primary samples of patients carrying different PAX5 translocations when compared to BCP-ALL cases with wt PAX5, as shown by microarray analyses and additionally verified by RQ-PCR. In addition to Lck over-expression, PAX5/ETV6 in pre-BI cells dephosphorylates the inhibitory domain of LCK (LCK Y505) thus hyper-activating the LCK signaling. Since LCK is known to activate STAT5 signaling pathway in Ba/F3 cells, we performed phosphoflow analyses showing the hyper-phosphorylation of STAT5 after IL7 stimulation. Downstream in the pathway, we demonstrated the up-regulation of cMYC and CCND2 by RQ-PCR analyses. Moreover PAX5/ETV6 positive pre-BI cells showed an increase in the replicative S phase cells and faster proliferation rate, likely due to a different STAT5 activation profile. The LCK inhibitor BIBF1120 reduced STAT5 phosphorylation in PAX5/ETV6 cells, thus decreasing the fraction of S phase cells and leading to a proliferation rate comparable to the control cells. These analyses sustains the role of PAX5 fusion genes in the over-expression of LCK and in the activation of signaling pathways alterative to the BCR to sustain the survival of leukemic cells. The further comprehension of the signaling mechanisms activated by PAX5 fusion genes will be fundamental for the development of new strategies to arrest tumor proliferation.

C004

GSX2 DEREGULATION IN CD7+ ACUTE MYELOID LEUKEMIA BEARING 4q12 TRANSLOCATIONS WITHOUT FUSION GENES

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Introduction. CD7 positivity (CD7+) is found in approximately 30% of AML which are assumed to arise from an early precursor maintaining myeloid and T-lymphoid potential (Norton, Leukemia 1987; Kurtzberg, Blood 1989). One of several chromosome translocations associated with CD7 expression (Harada, Br J Haematol 1995; Cools, Blood 2002; Jeandidier, Cancer Genet 2012) was the rare t(4;12)(q12;p13) (estimated incidence <1% adult AML), characterized by immature phenotype and poor clinical outcome (Harada, Br J Haematol 1995). In t(4;12)(q12;p13) 12p13 breakpoints fell within the ETV6 gene; 4q12 breakpoints involved either CHIC2 or no genes. Whatever genomic site 4q12 recombined with, it appeared to trigger ectopic GSX2 expression (Cools J, Blood 2002). GSX2, a homeobox gene and key regulator of neurogenesis, is normally expressed in neural stem cells in adults (López-Juárez, Genes Dev. 2013). *Methods.* FISH and qPCR studies were performed on bone marrow cells from 3 patients with AML-M0 and a 4q12 breakpoint. All had small pseudo-lymphoid blasts (Matutes E Eur J Haematol 1987), agranular basophilic cytoplasm and CD7 expression. The 4q12 and 17q22 regions were investigated with LSI 4q12 Tricolor Rearrangement Probe (Vysis, Abbott Molecular), homebrew BAC and fosmids. A break apart FISH assay (RP11-434C1 for the 5'; RP11-418C2+RP11-297N18 for the 3') investigated ETV6 at 12p13 in patient 1. qRT-PCR was performed using Light

Cycler 480 (Roche) and TaqMan assay probe (Applied Biosystems) Hs00370195_m1 for GSX2 gene. ABL1 (Hs00245445_m1) was the endogenous reference control. GL15 cell line RNA was our positive control (http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Hs.371899). In silico analysis used the UCSC database. *Results.* Breakpoints at 4q12 fell about 15 kb centromeric to GSX2 in patient 1, 50kb telomeric in patient 2 and within the fosmid G248P8717F7 encompassing GSX2 in patient 3. Translocation partners were ETV6 (12p13) in patient 1 and MSI2 (17q22) in patients 2 and 3. GSX2 was significantly over-expressed in all cases compared with healthy donors and with a group of CD7+ AML without 4q12 involvement. *Conclusions.* GSX2 deregulation derived from 4q12 recombining with ETV6 or MSI2. This is the first report of t(4;17)(q12;q22) with a MSI2 rearrangement, although MSI2 was involved in t(3;17) in myeloid malignancies with EVI1 overexpression (De Weer, Haematologica 2008). Interestingly, haematological features of our 3 cases suggested that GSX2 ectopic expression delineates a genetic subgroup in CD7+ AML. As FISH assay with the LSI commercial probes reliably picks up any 4q12 molecular breakpoint, we suggest using it in the diagnostic work-up to precise diagnosis of CD7+ AML not otherwise categorized. Identifying 4q12-related GSX2 de-regulation might fine-tune prognostic stratification of this AML subgroup.

C005

SETBP1 MUTATIONS IN 106 PATIENTS WITH THERAPY-RELATED MYELOID NEOPLASMS

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Introduction. Therapy-related myeloid neoplasms (t-MN) are myeloid disorders, including acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS) developing in patients treated with radiotherapy and/or chemotherapy for cancer or autoimmune diseases. SET binding protein 1 (SETBP1) has been recently reported as frequently mutated in chronic myelomonocytic leukaemia, atypical chronic myeloid leukaemia, secondary acute myeloid leukaemia and in distinct subgroups of primary myelodisplastic syndromes (RAEB1 and RAEB2). SETBP1 germline mutations are responsible of the Schinzel-Giedion syndrome. It is a congenital disease characterized by a higher prevalence of tumors, severe midface hypoplasia, congenital heart defect and skeletal anomalies. SETBP1 mutations were also been associated to chromosomes 7 alterations and SRSF2 point mutations, frequent in t-MN patients. *Aims.* Little is known regarding prevalence, clinical and prognostic role of SETBP1 mutations in t-MN. The aim of our study was to determine the frequency of SETBP1 mutations in t-MN patients. *Methods.* The patient cohort included 106 t-MN patients. Karyotype was abnormal in 52 of 81 (64.2%) patients with available karyotype. Chromosome 7 alterations were present in 16 of 81 (19.7%) patients. Mononuclear cells (MNCs) were separated from patients' BM at the time of initial diagnosis by Ficoll gradient centrifugation. DNA was extracted using a QIAamp DNA Mini Kit (Qiagen). Detection of SETBP1 mutations was performed by Sanger sequencing using the BigDye Terminator v 3.1 cycle sequencing kit (Life technologies). t-MN patients had been previously tested for DNMT3A, IDH1, IDH2, SRSF2, U2AF1 and SF3B1 mutations. Paired T-test was performed to test for association between mutations of SETBP1 and other genes, and with patient's karyotype. *Results.* The frequency of SETBP1 point mutations in the SKI-homologous domain was very low in our t-MN patient cohort (3/106; 2.83%). Two patients carried a G870S (COSM1234973) mutation, whereas there was one S869R mutation. The two SETBP1 G870S mutated patients were also mutated for the SRSF2 gene at position P95, whereas the carrier of S869R resulted wild type for SRSF2. No other associations between SETBP1 mutations, spliceosome machinery (SF3B1 exons 13-16 and U2AF1) and epigenetic regulators (IDH1 R132, IDH2 R140 and R172 and DNMT3A R882) somatic mutations were found. All patients mutaded in SETBP1 gene had developed a therapy-related MDS (one RAEB1 and two RAEB2). None of the therapy-related AML patients resulted mutated. In our t-MN patients, SETBP1 mutations were not associated to chromosome 7 abnormalities. *Conclusions.* Testing the largest t-MN cohort reported to date, we found a low incidence of SETBP1 mutations, suggesting that other major changes, such as complex and monosomal karyotypes, together with TP53 mutations, may play the major role in therapy-related leukemogenesis.

C006

B CELL-ACTIVATING FACTOR (BAFF-R) IS HIGHLY EXPRESSED IN B-LYMPHOID LEUKEMIC CELL LINES AND IN PRIMARY CHILDHOOD BCP-ALL SAMPLES

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Introduction. B cell-activating factor (BAFF) and its close relative proliferation-inducing ligand (APRIL) belong to the tumor necrosis factor (TNF) family. BAFF mediates the behavior of most B cells through interactions with their family receptors. Among them, only the BAFF receptor (BAFF-R) interacts specifically with BAFF, being the main responsible for primary B cell survival, selection and differentiation, in physiology and in disease conditions. We aimed to investigate the potential role of BAFF/BAFF-R axis in Acute Lymphoblastic Leukemia involving B-Cell Precursor (BCP-ALL). With this purpose, we analyzed the pathway in hematological tumor cell lines as well as in primary BM and PB samples from children affected by BCP-ALL. *Methods.* RT-PCR assays have been developed to determine BAFF and BAFF-R expression in cell lines. We implemented flow cytometry analysis to assess BAFF-R expression on blast cells in BM and PB diagnosis and follow-up samples of BCP-ALL patients. ELISA analysis has been used to valuate BAFF concentration in plasma samples. *Results.* Preliminary results demonstrated that BAFF-R is highly expressed in the B-lymphoid leukemic cell lines, such as REH, TOM1 and NALM-6 (RT-PCR analyses). Its expression is also detectable, although at lower levels, in mixed lymphoid/myeloid phenotype cell lines (such as THP1 and RS4;11), in myeloid K562 cells as well as in U937 histiocytic lymphoma cell lines. Supported by this data, we further collected BM and/or PB of 26 consecutive diagnostic samples of Pediatric BCP-ALL. We analyzed BAFF-R expression by flow cytometry, by indirect staining using a biotinylated antibody anti-BAFF-R and which was revealed by PE conjugated streptavidin antibody. In the same sample we assessed the CD19, CD10 and CD45 direct staining to recognize leukemic blast cells among the residual of normal cells. For each patient, in addition to the diagnostic sample, we analyzed at least one follow up sample (*i.e.* at day+8 or +15). We detected high levels of BAFF-R on CD19+CD10+CD45dim leukemic cells, which persisted during the follow up treatment. Moreover, we separated plasma by centrifugation of n=16 patients to analyze BAFF cytokine levels by ELISA technique, both in BM and PB samples. More interestingly, the ratio of BAFF level (ng/ml) over the number CD19+ blast cells (n°/mmc) revealed that the cytokine is consumed by blast cells at diagnosis and its level reaches physiological threshold after leukemic cell clearance, in follow up samples. *Conclusions.* We assessed the expression of BAFF-R on leukemic blast cells of BCP-ALL patients. Of note, its expression is maintained on residual tumor cells during the early drug treatment, suggesting the potential targeting of this molecule in future advanced treatment approaches. All together, BAFF/BAFF-R axis could have a role in BCP-ALL, although additional studies are required to comprehend its role in the pathogenesis of leukemia.

C007

SCREENING OF TP53 (TUMOR PROTEIN 53) MUTATIONS IN ADULT ACUTE MYELOID LEUKEMIA (AML) PATIENTS: STRONG ASSOCIATION WITH COMPLEX KARYOTYPE AND POOR OUTCOME

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Introduction. AML is a heterogeneous disease with various chromosomal aberrations. The karyotype at diagnosis provides important prognostic information that influences therapy and outcome, and patients (pts) with complex karyotype (CK-AML) have generally a poor outcome. TP53 is the most frequently mutated gene in human tumors. The reported TP53 mutation rate in AML is low (2.1%). In contrast, the incidence of TP53 mutations in AML with a complex aberrant karyotype (CK-AML) is higher (69-78%). *Aims.* To investigate the frequency and

the prognostic role of TP53 mutations in adult AML pts focusing the screening on subgroups of pts with chromosome abnormalities. *Methods.* 106 adult AML pts with FAB-M0, M1, M2, M3, M4, M5, miscellaneous cytogenetic abnormalities and normal karyotype (nK-AML, 14/106 pts) were examined. Forty-four pts (41.1%) showed 3 or more chromosome abnormalities (CK-AML), 42 (39.6%) presented one or two cytogenetic abnormalities (other-AML) and in 6 cases the karyotype was not available. Genomic DNA and/or cDNA were isolated from mononuclear AML blast cells. TP53 mutation screening was performed on all 106 AML pts, in particular in 42 from exon (ex) 2 to 11, in 48 from ex 4 to 11 and in 16 pts from ex 2 to 8. Analysis was focused on coding sequences (RefSeq GRCh37/hg19 NG_017013.2). *Results.* By PCR and subsequent Sanger sequencing, mutations of TP53 were detected in 23 cases (21.1%). Seven pts revealed 2 mutations. 83% of all mutated pts had CK (19/23) by contrast the frequency of mutations was very low in "no CK-AML" pts (6.5%). Overall, mutated patients included 19/44 with CK-AML (43.2%); 1/6 (16.7%) with nK and 3/42 (7.1%) with other-AML. 26 TP53 point mutations and 4 TP53 deletions were found. R248W and M237I mutations were found in 2 pts each. Interestingly, one patient showed a base substitution in the exon 5 donor splice site and a frameshift deletion in the transcript Δ126-132. Twenty-six out of 30 mutations/deletions (86.6%) were located in the DNA binding domain, 2 in the carboxyl-terminal tetramerization and regulatory domains and 2 in the transcriptional activation domain. All mutations in coding regions were classified by the IARC database (http://p53.iarc.fr/TP53GeneVariations.aspx) as deleterious. Of note, alterations of TP53 were significantly associated with poor outcome in terms of both overall survival and disease free-survival (P <0.0001) (Figure 1). *Conclusions.* Our data demonstrated that TP53 mutations occur in 21.7% of AML with a higher frequency in the subgroup of CK-AML (p< 0.0001–Fischer's exact test). Since TP53 mutations have predicted to be deleterious and significantly correlated with prognosis, TP53 mutation screening should be recommended in CK-AML pts.

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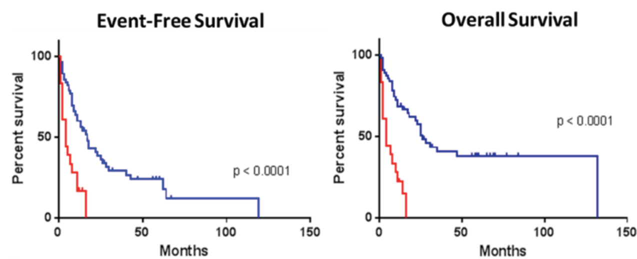

Figure 1. Event-Free Survival and Overall Survival in *TP53* wild type (blue line) and in *TP53* mutated (red line) AML patients.

C008
PROPOSAL OF MINIMAL MORPHOLOGICAL CRITERIA FOR DEFINING BONE MARROW DYSPLASIA 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 (BM) 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. The aims of this study were to identify minimal reproducible morphological criteria to define marrow dysplasia useful for a correct application of WHO classification and to evaluate the prognostic relevance of the degree of dysplasia. *Methods.* We retrospectively examined the cytological features of BM smears from 318 MDS cases, 203 patients with non-clonal cytopenia and 74 healthy subjects (learning cohort). By counting 100 cells for the erythroid and granulocytic lineages and at least 30 megakaryocytes and classifying them for their dysplastic changes,we developed a morphological score for MDS identification on the basis of the discriminant power of single morphological abnormalities. The diagnostic value and reproducibility of the proposed criteria were tested in a validation cohort. *Results.* We identified the most discriminant morphological features for dyserythropoiesis (megaloblastosis, multinuclearity, nuclear lobulation, pyknosis, defective hemoglobinisation, cytoplasmic fraying, ring sideroblasts), dysgranulopoiesis (increased myeloblasts, Auer rods, hypolobulation, abnormal nuclear shape, hypogranulation) and dysmegakaryopoiesis (micro-, monolobar or small binucleated megakaryocytes, megakaryocytes with multiple separated nuclei). For each parameter the optimal cut-off value to discriminate between MDS and controls and the weight in the recognition of BM dysplasia were determined to develop a score for defining minimal morphological criteria for MDS. This score showed high sensitivity and specificity (>90%). BM aspirates from a validation cohort of 157 MDS cases and 43 controls with non-clonal cytopenia were independently evaluated by two expert panels who applied the morphological score. The interoperator agreement was .82 by K test (.75 for patients without excess blasts, .87 for patients with excess blasts). Erythroid score value did not significantly affect survival, while granulocytic or megakaryocytic score levels had a significant effect on OS (P<.001). Also multilineage dysplasia showed an independent unfavorable prognostic value (P<.001). A close association was found between ring sideroblasts and SF3B1 mutations (P<.001) and between severe granulocytic dysplasia and mutations of ASXL1, RUNX1, TP53 and SRSF2 genes (P from .03 to .001). *Conclusions.* Our morphological score that improves the objectivity and reproducibility of microscopic analysis may be useful in the work-up of patients with suspected MDS. Prognostic systems including the evaluation of the degree of BM dysplasia should be adopted for clinical decision-making.

Chronic Lymphoproliferative Disorders I

C009

LOW-DOSE LENALIDOMIDE IMPROVES CHIMERIC ANTIGEN RECEPTOR-BASED IMMUNOTHERAPY IN CLL BY REVERTING T-CELL DEFECTS *IN VIVO*

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Introduction. Lenalidomide is an immunomodulatory agent (IMID) able to induce significant long-lasting responses in Chronic Lymphocytic Leukemia (CLL) patients. Lenalinomide was found to modulate CLL tumor microenvironment through down-regulation of critical cytokines, activation of immune effector cells and to revert defects in immunological synapse between T and CLL cells. Chimeric antigen receptors (CARs) are emerging as a powerful tool to redirect T-cell specificity against leukemia. In order to revert *in vivo* the acquired T cell defects in CLL patients, it becomes very intriguing to explore a CAR-based immunotherapy combined with low doses of lenalidomide, to maximize the effect of the immune attack. *Methods.* Using the Rag2-/-γ≥c-/--xenograft model of human CLL we performed experiments where mice were injected with CAR.CD23+T cells from CLL patients together with lenalidomide at low concentrations, uneffective in monotherapy. *Results.* We observed a decreased percentage of CD19+leukemic cells in all lymphoid and non-lymphoid tissues of mice after 20 days of treatment, as compared to controls treated with CAR.CD23+T cells or lenalidomide alone. This combination resulted also in improved survival of the treated cohort (NT+lenalidomide *vs* CAR+lenalidomide: $p<0.03$, n=7). The effect of the combination with low dose lenalinomide was more effective also when compared to the addition of hIL-2 as in traditional settings. In accordance to the *in vivo* efficacy, CAR.CD23+T cells were observed in all leukemic sites suggesting an ability to migrate and home *in vivo*. Moreover, CD23.CAR+T cells purified from bone marrow were still able to mount a tumor specific cytotoxic response *in vitro*, reaching more than 50% of tumor lysis in both the conditions with lenalidomide and hIL-2, compared to 20% of tumor lysis exerted by unmanipulated T cells. *Conclusions.* These results conceivably support the use for CLL immunotherapy of low doses lenalidomide to improve CAR cytotoxic response and avoid the potential impairment of an effective immune response.

C010

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

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Human SLAMF-1 (signaling lymphocytic activation molecule) is expressed on hematopoietic cells where it acts as a co-stimulatory molecule through self-interactions. Moreover, it performs as a microbial sensor, regulating bacterial phagosome functions through an ubiquitous cellular autophagic machinery. We investigated the role of SLAMF-1 in human B cells, exploiting chronic lymphocytic leukemia (CLL) as a model. CLL is characterized by the expansion of a monoclonal population of mature B lymphocytes, with a highly variable clinical course. We demonstrated that SLAMF-1 is expressed at variable levels by CLL cells and marks the subset characterized by a good prognosis. Silencing of SLAMF1 expression in Mec-1 cell line led to a down-modulation of pathways connected to cell death, intracellular vescicle formation and recirculation, as determined by global gene expression analysis. Moreover, the apoptotic response to fludarabine treatment observed in control Mec-1 cells (constitutively SLAMF-1+) was completely lost in silenced cells. Consistent with previous findings, fludarabine activated autophagy in the Mec-1 cells. However, in SLAMF1- cells no modulation of the autophagic flux was highlighted, suggesting that deletion of the molecule is responsible for this phenomenon. In line with this finding, fludarabine responses were different in CLL patients divided according to SLAMF-1 expression, as well as in SLAMF-1high *vs* low cells separated through cell sorting from the same patient. Functional experiments confirmed that the engagement of the receptor initiates a signaling cascade that involves the direct interaction with the adaptor molecule Eat-2 and that converges on the activation of the MAP kinases. Prolonged engagement of SLAMF-1 led to the appearance of autophagic vesicles and to the increase of LC3B formation. The modulation of autophagy was mediated by ROS and by the sequential phosphorylation of Jnk1/2 and Bcl-2: the final result was the release of Beclin-1 from Bcl-2 and the consequent assembly of the autophagic complex, including Vps34. Taken together, these results suggest that SLAMF-1 could represent a novel marker for the subset of CLL patients with an indolent clinical course. These results also suggest a link between the activation of the autophagic process and a milder form of the diseae, with a better response to fludarabine treatment.

C011

INDUCTION OF MICRORNA 15 AND 16 REPRESENTS A POTENTIAL TARGETED THERAPY IN CHRONIC LYMPHOCYTIC LEUKEMIA

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More than 50% of Chronic Lymphocytic Leukemia (CLL) have deletions in chromosome 13 [del(13q14)] with the loss of expression of microRNA (miR)15 and miR16. We analyzed by miRNAome the expression of miR15 and miR16 in the cells from a cohort of 203 early stage CLL cases (O-CLL1, NCT00917540). We found that miR15 and 16 were down-regulated in 95 CLL cases with del13q14 compared to 108 CLLs lacking del13q14, even only for miR16 was statistically different ($p<0.0001$). Moreover, the expression of miR15 and 16 was significantly lower in the 9 biallelic del13q14 CLL patients *versus* 68 monoallelic cases ($p=0.001$ and $p<0.0001$ for miR15 and 16, respectively). The remaining 18 patients with mixed biallelic/monoallelic del13q14 cells showed an intermediate level of miR-15 and 16. Based on these observations we first performed *in vitro* experiments in which miR15 and 16 mimics or miR neg ctr were transfected by electroporation in 10 biallelic del13q14 CLLs. MiR15 and 16 induction was evaluated by qPCR and by smartflare technology and flow cytometry. After 48 hrs culture we demonstrated the induction of apoptosis and down-modulation of BCL2, MCL1 and cyclin D1/D2 proteins in both transfected miR15 and 16 compared to miR neg ctr ($p<0.005$). To further investigate the potential therapeutic effect of miR15 *in vivo* we used a xenograft model generated by infusion of primary CLL cells into immunodeficient NSG mice. In order to improve engraftment of CLL cells, PBMC from biallelic del13q14 patients were stimulated for 3-5 days with CD3/CD28 beads and rIL-2 to allow activation of autologous T cells essential for leukemia cells to successfully engraft. 30-50x106 cells were inoculated in NGS mouse by intravenous injection

(IV) and disease progression was monitored by analyzing PBMC cells for the presence of circulating CLL cells CD19/CD5+ by flow cytometry and by Magnetic Resonance Imaging (MRI) to evaluate the size and the integrity of the spleen by using USPIO nanoparticles as contrast reagent. Mice were then subdivided in two groups that were treated by three injections, at intervals of 48 hrs, of miR15 or miR neg ctr complexed with invivofectamine Reagent. Three days after the last treatment, animals were sacrificed and paraffin-embedded spleen slices were stained with anti CD20, CD3, CD4, CD8, BCL2, CD20/Ki67 antibodies. Control mice showed the presence of CLL cells localized in typical focal aggregates in the spleens. In contrast, mice treated with miR15 showed regression of the focal aggregates, fibrous areas previously occupied by the neoplastic clone, residual CD20-positive B cells displaying low BCL-2 and ki67 proliferation marker expression. Conversely the presence of numerous CD4/ ki67 proliferating T cells widespread in the spleen was observed. All together, these results suggest that induction of miR15 or 16 triggers the apoptotic pathways in biallelic del13q14 CLL cells representing a possible target therapy at least for this subgroup of CLL patients.

C012

LENALIDOMIDE INDUCES A PRO-INFLAMMATORY PHENOTYPE IN NURSE-LIKE CELLS DERIVED FROM CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction. Lenalidomide is an immunomodulatory agent clinically active in CLL patients. The specific mechanism of action is still undefined, but includes the modulation of microenvironment. In CLL patients, nurse-like cells (NLCs) differentiate from CD14+ mononuclear cells and nurture/protect CLL cells from apoptosis. NLCs resemble M2 macrophages with potent immunosuppressive functions. We investigated whether lenalidomide may interfere with the nursing and protective phenotype of NLCs. *Methods.* NLCs were generated in presence or absence of lenalidomide: cell surface markers, phagocytosis and induction of T cell proliferation were analyzed after 10 days. NLCs activation was measured using a yellow tetrazolium MTT assay after 5 days of culture and NLCs proliferation was measured by CFSE staining. Microarray-based gene expression profiles of NLCs treated or not with lenalidomide were evaluated after 10 days and data were confirmed by real time PCR. *Results.* Lenalidomide modifies the immunophenotype and the biological characteristics of NLCs. First, treatment with lenalidomide 0.5μM and 1μM increased the number of NLCs to 268% and 309% compared to untreated control (100%) respectively ($p<0.05$). To explain the high number of NLCs generated by lenalidomide, we analyzed cell activation and proliferation. We observed a strong increase in NLCs activation after treatment with lenalidomide that correlated with stimulation of NLCs proliferation from 44% to 55% (% of dividing cells) ($p<0.05$). In contrast with the high number of NLCs generated in presence of lenalidomide, we found that NLCs lost the ability to nurture and protect CLL cell from apoptosis reducing their viability from 54.2% to 44.5% ($p<0.05$), but they strongly attracted CLL cells reaching an increase of adhesion to 227% and 212% with the addition of 0.5 and 1μM lenalidomide ($p<0.05$). Accordingly with these results, we investigated the ability of lenalidomide to interfere with leukemia-promoting activity of NLCs. Lenalidomide improved the ability of NLCs to engulf zymogen particles to 141% and 155% with 0.5μM and 1μM compared to control ($p<0.05$), further confirmed analyzing the uptake of FITC-dextran by NLCs that increased to 252% and 356% in presence of 0.5 and 1 μM dose compared to untreated control ($p<0.01$). Moreover lenalidomide strongly improved the ability of NLCs to induce T cells proliferation from 19.5% to 35% (% of dividing cells) ($p<0.05$) (Figure 1). In addition, gene expression profiling showed a switch to a pro-inflammatory profile of NLCs induced by treatment with lenalidomide characterized by high IL-2, low IL-10 and low CD163. *Conclusions.* Collectively, our data provide new insights into the mechanism of action of lenalidomide that reverts NLCs polarization from M2 to M1-skewed phenotype affecting the supporting and protective microenvironment generated by CLL into tissues.

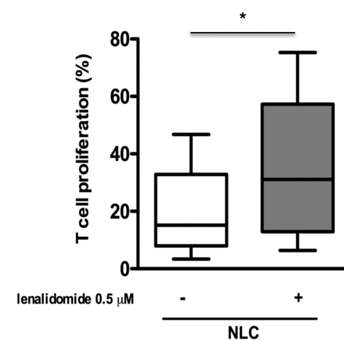

Figure 1.

C013

STAT3 IS CONSTITUTIVELY ACTIVATED IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA CELLS AND REPRESENTS A POTENTIAL THERAPEUTIC TARGET

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Introduction. Chronic Lymphocytic Leukemia (CLL) is characterized by the accumulation of B lymphocytes due to both intrinsic defects of the leukemic cell and to extrinsic microenvironmental factors that contribute to the acquisition of the transformed phenotype and the spread of the disease. Several molecules, released by microenvironment partners (*i.e.* Mesenchymal Stromal Cells, MSCs), have been shown to signal through JAK (Janus kinases)-STAT (signal transducers and activators of transcription) pathways thus favoring cell survival. STAT3 plays a key role in supporting cell survival by promoting the expression of anti-apoptotic factors such as Mcl-1 and Bcl-2, both overexpressed in CLL. With this as a background, we were aimed at studying STAT3 role in CLL pathogenesis. *Methods.* STAT3 phosphorylation (Ser727 and Tyr705) was analyzed in CLL and normal B cells by western blotting and FACS analysis using phospho-specific antibodies. Subcellular localization of STAT3 was observed by confocal microscopy. JAK/STAT3 inhibition was assessed by AG490 cell incubation followed by Annexin V/Propidium Iodide test. CLL B cells were also cultured onto a stromal layer represented by CLL-MSCs. Dasatinib, an inhibitor of Lyn, was used to assess Lyn involvement in the cross-talk with JAK/STAT3 axis. *Results.* The study of STAT3 activation pattern in CLL cells demonstrated a constitutive activation in both Ser727 and Tyr705. STAT3 constitutive phosphorylation at Tyr705, essential to its activation and here described for the first time in CLL, was increased following co-culture of leukemic cells with CLL-MSCs, thus supporting the hypothesis that CLL stroma sustains STAT3 activity. Cultures of CLL cells with AG490, an inhibitor of JAK2-3 that in turn is an established activator of STAT3 at Tyr705, demonstrated that this compound was able to induce apoptosis in CLL B cells by affecting the activation state of STAT3. Similar results were obtained when CLL cells were co-cultured in presence of MSCs, demonstrating the ability of AG490 to bypass the effects of the pro-survival stimuli coming from MSCs. STAT3 Tyr-phosphorylation may be also related to Lyn kinase, thus providing evidence for the discovery of an alternative pathway for STAT3 activation. The Lyn established overexpression and constitutive activation in CLL B cells in fact would account for the constitutive phosphorylation of STAT3 at Tyr705 observed even when leukemic cells are not under the influence of the microenvironment. In this context, our preliminary data demonstrate that Lyn inhibition by Dasatinib inactivates STAT3. *Conclusions.* Bypassing the pro-survival stimuli provided by the tumor microenvironment, the ability of AG490 to induce apoptosis in leukemic B cells offers a starting point for the development of new therapeutic strategies in CLL. These studies also provide new insights for the study of the pathogenesis of CLL focusing the attention on the cross-talk between JAK/STAT and BCR/Lyn axes.

C014

ROLE OF HSP70 IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA CELLS SURVIVAL

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Introduction. B-cell chronic lymphocytic leukemia (B-CLL) is characterized by the accumulation of B lymphocytes due to proliferative activity and to apoptosis resistance. Although innovative treatments have shown a positive impact on prognosis and clinical outcome, some patients relapse or become unresponsive to common therapies. One of the main goal of the research in this field is the identification of molecules involved in the pathogenesis and which might be targeted to develop new therapeutic strategies for CLL. Using Reverse Phase Protein Array technique we previously demonstrated that Heat shock protein of 70kDa (HSP70) was significantly overexpressed in CLL *vs* normal B cells. Considering the anti-apoptotic and protective role of HSP70 in cancer, we were aimed at better characterizing the role of this protein within the pathogenetic mechanisms leading to CLL. *Methods.* HSP70 proteic level was analyzed by western blotting analysis in B cells from 42 CLL patients and 11 healthy subjects. HSP70 levels were correlated to IGHV mutational status and karyotype. HSP70 was also analyzed in leukemic B cells from patients before and after Fludarabine or Bendamustine based regimens. Confocal microscopy was used to investigate subcellular localization of HSP70 and its co-chaperone BAG3. Finally, the effects of HSP70 inhibition (by Pifthrin-μ, MKT-077, and VER155008) were evaluated by Annexin V/Propidium Iodide flow cytometry test. *Results.* We found that HSP70 protein was overexpressed in leukemic *vs* normal B cells and correlated to poor prognosis. In fact, IGHV unmutated or high risk karyotype (17p-, 11q- or 12+) patients presented higher levels of HSP70 with respect to patients with a favorable prognosis. We also observed that HSP70 levels decreased in those patients who were responsive to *in vivo* chemotherapeutic regimens while HSP70 was unchanged, or even increased, in unresponsive patients, thus hypothesizing the involvement of HSP70 in drug-resistance mechanisms. By confocal microscopy, we observed an abnormal nuclear localization of HSP70 and of its co-chaperone BAG3 in leukemic cells. Considering the pro-survival role played by HSP70, we analyzed the effects of its inhibition in leukemic cells of our patients by using different HSP70 inhibitors, *i.e.* Pifthrin-μ, MKT-077, and VER155008. All three inhibitors have been proven to be effective in inducing a significant reduction in cell viability of CLL B cells. *Conclusions.* These findings suggest a pivotal role for HSP70 in the regulation of cell survival of leukemic B cells and hint that this protein might represent a target for the development of new therapeutic strategies in CLL.

C015

THE PI3K-δ INHIBITOR TGR-1202 IN COMBINATION WITH BRENTUXIMAB VEDOTIN (SGN-35) SYNERGISTICALLY INDUCES G2/M PHASE ARREST AND CELL DEATH IN HODGKIN LYMPHOMA CELL LINES

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Introduction. The PI3K pathway is consistently activated in relapsed/refractory Hodgkin lymphoma (HL), suggesting that TGR-1202, a novel inhibitor of the delta isoform of PI3K (PI3K-δ), might represent an attractive therapeutic option. The anti-CD30 monoclonal antibody Brentuximab Vedotin (BV) conjugated to the microtubule-disrupting agent monomethyl auristatin E (MMAE) has recently been reported to induce an overall response rate of 75% in relapsed/refractory HL, but is associated with limited response duration. Combination therapies aimed at enhancing the anti-tumor activity of BV and reducing its side effects may have significant clinical impact in the treatment of relapsed/refractory HL. Our study was aimed at investigating the activity and mechanism(s) of action of TGR-1202 in combination with BV in non-clinical models of HL. *Methods.* Three HL cell lines (L-540, KM-H2 and L-428), were used to test the effects of TGR-1202 and/or BV by means of *in vitro* assays analyzing cell growth and cell death. Additionally, WB and immunofluorescence were used to assess modulating effects of TGR-1202 and/or BV on microtubule interacting proteins, PI3K/AKT as well as apoptosis pathways. The efficacy of TGR-1202/BV combination was finally confirmed in NOD/SCID mice with HL cell line xenografts. *Results.* TGR-1202 in combination with BV synergistically inhibited the mean (±SEM) growth of HL cells (TGR-1202: -40±4%; BV: -30±2%; TGR-1202/BV: -85±1%). This finding was associated with a 4-fold increase of G2/M phase, a 3-fold reduction of cells in S phase, and a marked Cyclin B1 and p21 overexpression. Upon TGR-1202/BV treatment, cell death values were increased 3-fold over single agents (TGR-1202: 27±2%; BV: 27±2%; TGR-1202/BV: 75±2%). Analysis of caspase-3 and PARP cleavage and blocking experiments with the pan-caspase inhibitor Z-VAD-FMK revealed a caspase-dependent cell death mechanism. In addition, the anti-lymphoma effects of TGR-1202 were associated with a marked time-dependent inhibition of PI3K/Akt pathway and dephosphorylation of GSK-3β, Aurora kinases, and Stathmin, suggesting that modulation of molecules associated with microtubule polymerization are critically involved in TGR-1202/BV-triggered cell death. The combined TGR-1202/BV treatment resulted in a potent tubulin polymerization inhibition and disruption of the mitotic spindle integrity. *In vivo* TGR-1202/BV treatment significantly reduced the growth of L-540 nodules, resulting in an 55% tumor growth inhibition ($P \leq .0001$) compared to single agents, in the absence of any toxicity. Interestingly, as compared to controls or single agents, TGR-1202/BV significantly increased *in vivo* microtubule disruption, resulting in a marked tumor necrosis (3-fold increase, $P \leq .0001$). *Conclusions.* TGR-1202 enhances the anti-tumor activity of BV by increasing drug-induced apoptosis and tubulin disruption in HL cells. Our data provides a strong rationale for evaluating TGR-1202/BV in relapsed/refractory HL patients.

C016

ANALYSIS OF THE ROLE OF PROTEIN KINASE CSNK2 DOWNSTREAM OF B-CELL RECEPTOR

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Introduction. Normal and malignant B-lymphocytes depend on the B cell receptor (BCR) for their survival, development and activation. BCR is an antigen (Ag)-binding membrane immunoglobulin (mIg) complexed with an Igα/Igβ transmembrane heterodimer. Ag engagement leads to BCR cross-linking and through a cascade stimulating several molecules, such as Lyn, Syk, PI3K, Btk BLNK, etc. it ends up in the activation of specific and context-dependent transcription factors, like NF-κB, NF-AT, c-Myc, Bcl-6 and IRF4. CK2 is a tetrameric serine-threonine kinase consisting of two catalytic (α) and two regulatory (β) subunits, crucial for cell differentiation, proliferation and survival. It takes part in several hemo-lymphopoiesis-regulating pathways, like the Wnt/β-Catenin, NF-κB, JAK2/STAT3 and PI3K/AKT/mTOR and promotes a "non-oncogene addiction" phenotype in tumor cells. Our and other groups have shown that CK2 is overexpressed and overactive in a wide variety of B cell-derived tumors, such as acute lymphoblastic leukemia, chronic lymphocytic leukemia, mantle cell lymphoma and multiple myeloma. *Methods.* To elucidate the physiological and pathogenic role of CK2 in B-lymphocytes, we have generated CK2β B cell specific conditional knockout (KO) mice (CK2βflox/flox; CD19-Cre/+). CK2β expression was analyzed in the CD19+ fraction of spleen and bone marrow, by Western blot and Real time qPCR. Splenic B cells were obtained by EasySep Mouse B Cell Isolation Kit (Stemcell). FACS analysis of B-cell populations was performed with markers for marginal zone (MZ) and follicular (FOB) B cells. Antibodies were from Beckton-Dickinson (BD); acquisition with FACS Calibur or Aria, analysis with CellQuest or FlowJo software. To inhibit CK2 activity in cell lines, we employed CX-4945, a selective, ATP-competitive, orally bioavailable compound, with antitumor activity validated in solid tumor cell lines and murine xenograft models and currently under scrutiny in phase I clinical trials.

To stimulate the BCR we utilized an αIgM antibody (invitrogen). *Results.* CK2β KO mice displayed B-lymphopenia, hypogammaglobulinemia and a strong imbalance between MZ and FOB splenic B cells. Downregulation of CK2β was clear by RT-PCR and WB. Since a weak BCR signalling is known to cause reduced FOB and increased MZB, we checked CK2 downstream of BCR in CTR and CK2β KO CD19 B-lymphocytes and lymphoma cell lines treated with CX-4945. Calcium fluxes were markedly impaired in CK2β KO or CX-4945-treated B cells as compared to ctr. P-p65Ser529 and p-AktSer473 were markedly decreased when CK2 kinase activity was impaired. Upon BCR engagement of Raji NHL cells, CK2α relocalized from the nucleus to the cytosol with a very fast kinetics (Figure 1). *Conclusions.* CK2 is a critical component of B cell receptor signalling and pivotal for B cell development and function. Further studies are ongoing to elucidate its mechanistic role in physiology and pathobiology of B cells.

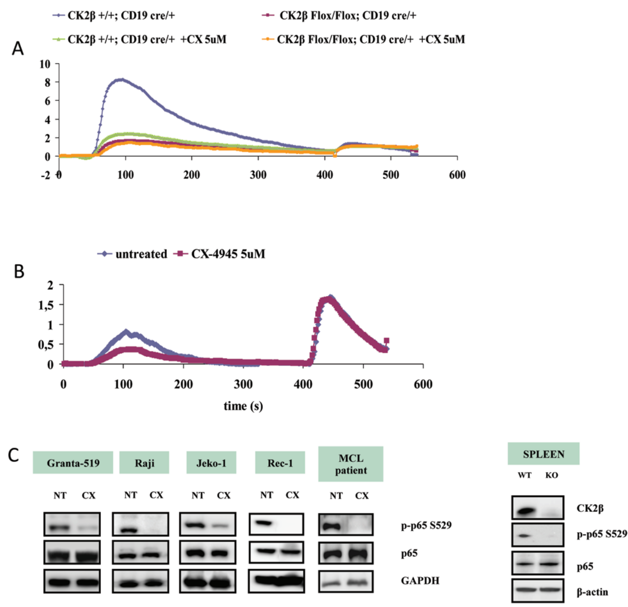

Figure 1.

Monoclonal Gammopathies and Multiple Myeloma I

C017

MIR-494 REDUCES CELL PROLIFERATION AND AFFECTS C-MYC EXPRESSION IN HUMAN MYELOMA CELL LINES

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Introduction. MicroRNA (miRNA) deregulation has been involved in the pathogenesis of plasma cell dyscrasia and associated with distinct genetic abnormalities occurring in the disease. In particular, we recently demonstrated (Lionetti *et al.*, CCR 2013) that several miRNAs are differentially expressed between patients with multiple myeloma (MM) and those affected by primary plasma cell leukemia (pPCL), thus potentially contributing to the aggressiveness of PCL. Among these, we focused on miR-494 found to be down-regulated in PCL. miR-494 appears to play differential roles in various tumors. miR-494 is considered an oncogenic microRNA that regulates the G1/S transition during liver tumorigenesis; however, miR-494 is down-regulated in human cholangiocarcinoma and gastric carcinoma, and its reinforcement results in cancer growth inhibition, suggesting that miR-494 acts as an anti-oncogene in a specific cellular context. The role of the miR-494 in MM cells remains to be elucidated. *Methods.* Human myeloma cell lines MM.1S and U266 were transfected with miR-494 mimic (Ambion) using the Neon technology (Life Technologies). Nontargeting miRNA mimic was used as negative control. Cells were collected and processed for quantitative real time PCR (Q-RT-PCR) (TaqMan miRNA assays, Applied Biosystems), immunoblotting (WB) and cell cycle analysis at different time points after nucleofection (24, 48, 72 and 96h). Cell cycle distribution was analyzed by flow cytometry after propidium iodide staining. *Results.* To investigate the role of miR-494 in myeloma cells, we transiently overexpressed miR-494 in MM.1S and U266 cell lines, which express low levels of this miRNA. miR-494 expression in transfected cells was significantly higher as compared to negative control at all time points but 96h analyzed by Q-RT-PCR. At 48/72h changes in cell number were observed in comparison to negative control. In addition, cell cycle analysis revealed that transfected MM.1S and U266 cells exhibited an increase in the percentage of cells in G0/G1 starting from 24h, becoming more marked at 48h and decreasing at 72/96h. As miR-494 negatively regulates c-myc expression in different cell types, we verified if this modulation could occur in our experimental model. WB analysis of whole cell lysates showed a pronounced reduction of c-myc protein expression in miR-494-transfected cells: based on densitometric analysis, c-myc levels were more than 70% lower than controls at 24h (40% lower at 48h), beginning to raise towards basal levels at 72h. *Conclusions.* Our preliminary functional data indicate that the miR-494 may act as putative tumor-suppressor in MM. Furthermore, in agreement with available evidence in other systems, it appears to modulate the expression of c-myc also in MM cells. These data provide a rationale for evaluating miR-494 as a possible target of miRNA-based therapeutic strategies in MM.

C018

RECONSTRUCTION OF MICRORNA/GENES TRANSCRIPTIONAL REGULATORY NETWORKS OF MULTIPLE MYELOMA THROUGH IN SILICO INTEGRATIVE GENOMICS ANALYSIS

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Introduction. The identification of deregulated miRNA in multiple myeloma (MM) has progressively added a further level of complexity

to MM biology. MiRNA are currently considered both emerging therapeutic targets and innovative intervention tools. Herein, we take virtue of in silico integrative genomics analysis to generate an unprecedented global view of the transcriptional and post-transcriptional regulatory networks modulated in MM and to define microRNAs impacting in regulatory circuits with potential functional and clinical relevance. *Methods.* miRNA and gene expression profiles in four large proprietary (Lionetti M, 2009, Blood; Wu P, 2013, Br J Hematol) and public (Gutierrez N, 2010, Leukemia; Zhou Y, 2010, Proc Natl Acad Sci) representative MM datasets, available from retrospective and prospective clinical trials and encompassing a total of 305 patients at diagnosis, were analyzed by means of MAGIA2 method (Bisognin A, 2012, Nucl Acid Res), to identify mixed circuits (triplets) involving miRNA/transcript/transcription factor (TF; http://gencomp.bio.unipd.it/magia2/). Micrographite computational procedure (Calura E, 2014, Nucl Acid Res), was applied to the integrated analyses on both miRNA and gene expression profiles, with the final aim of identifying highly modulated miRNA-gene networks in the disease, in terms of differential strength of inferred interactions. This approach grounds on gene networks whose topology is derived from pathways, which are enriched with miRNA–target gene interactions stemming from expression data analysis and from predicted and validated interactions from public databases (TargetScan, TarBase and miRecords). *Results.* Matched miRNA and genes/transcripts expression profiles of 4 MM dataset (including 40, 52, 60 and 153 cases) were combined with a curated set of miRNA-target predictions, thus allowing to identify critical circuits involved in MM, and the results obtained were overlapped to find common triplets. Two circuits emerged as common to at least two datasets, including hsa-mir-200c-3p, the TF ATF2 and the autophagy receptor NBR1, and the other hsa-mir-497-5p (that we have previously found associated with treatment response in plasma cell leukemia – Lionetti *et al.,* 2013, Clin Can Res), the Rho-like GTPase ARHGEF9 and the TF NFE2L1. Integrated pathway analyses performed on miRNA and gene expression data of the 153 MM patients included in MRC Myeloma IX trial, stratified according to their hyperdiploid (HD) status, allowed to generate a meta-pathway composed by the miRNA/genes and the interactions that mainly characterize HD in MM. *Conclusions.* Our analysis on 4 independent MM datasets allowed to define a preliminary but comprehensive picture of regulatory networks involving genes and miRNAs with putative roles in MM tumor biology, and identify specific elements and interactions thereof that are worth further investigation.

C019

TRANSCRIPTIONAL AND PROTEOMIC PROFILES OF BONE MARROW CD14+ CELLS IN MULTIPLE MYELOMA (MM) COMPARED TO SMOLDERING MM AND MGUS: OVEREXPRESSION OF INTERLEUKIN (IL)-21 RECEPTOR AND ITS INVOLVEMENT IN MM-INDUCED OSTEOCLASTOGENESIS

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Introduction. Multiple myeloma (MM) patients are characterized by bone marrow (BM) microenvironment alterations, as compared to patients affected by smoldering MM (SMM) and monoclonal gammopathy of uncertain significance (MGUS). The aim of this study was to analyze the transcriptional and proteomic profiles of the BM CD14+ cells across different types of monoclonal gammopathies, based on their primarily involvement in osteoclastogenesis, vasculogenesis and immune system. *Methods.* CD14+ monocytes were purified from a total cohort of 59 patients including 30 patients with symptomatic MM, 16 patients with SMM and 13 patients with MGUS. CD14+ cells were isolated from the CD138 negative fraction of patient BM samples by an immunomagnetic method with anti-CD14 mAb conjugated with microbeads (purity >94%). CD14+ gene expression profiles (GEPs) were evaluated by GeneChip HG-U133Plus 2.0 arrays (Affymetrix®) in 25 MM, 11 SMM and 8 MGUS. The proteomic analysis was performed on CD14+ cells of 5 MM, 5 SMM and 5 MGUS. Samples were run on Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific®) after labeling with tandem mass tags and data analyzed with Proteome Discoverer software. *Results.* Different types of GEP analysis were conducted. A multiclass analysis identified 18 differentially expressed genes in MGUS, SMM and MM. The comparison of MM with both SMM and MGUS samples identified 61 genes differentially expressed in CD14+ cells (37 up-regulated and 24 down-regulated). Interestingly, we found that CD14+ of MM patients, as compared to SMM and MGUS, over-expressed some cytokine receptors (IL21R and IL-15R), pro-osteoclastogenic chemokines (CXCL10 and CXCL11), interferon-inducible proteins (IFI27 and IFI44) and SLAMF7. By a proteomic approach, different CD14+ monocyte expression profiles were found comparing MM patients with MGUS and SMM ones. Interestingly, MM monocytes over-expressed proteins involved in cell adhesion and inflammation and down-regulated molecules implicated in antimicrobial functions. Further we investigated the potential role of IL-21R up-regulation in CD14+ cells. Firstly, any significant difference was not observed in the BM IL-21 levels between MM, SMM and MGUS in a large cohort of 160 patients. On the other hand, we confirmed that IL-21R was up-regulated at protein level in CD14+ of MM patients as compared to both SMM and MGUS; the conditioned media of MM cells up-regulated IL21R mRNA in CD14+ cells. The treatment with rhIL-21, at the concentration observed in BM plasma (30pg/ml), stimulated CD14+-derived *in vitro* osteoclastogenesis increasing number and size of osteoclasts, in MM patients but not in SMM and MGUS. *Conclusions.* Our results indicate that different expression fingerprints characterize BM CD14+ monocytes of patients with MM as compared to those with SMM and MGUS, including over-expression of IL-21R possibly involved in MM-induced osteoclast activation through an increased sensitivity to IL-21.

C020

HIF-1α INHIBITION BLOCKS THE CROSS TALK BETWEEN MULTIPLE MYELOMA PLASMA CELLS AND TUMOUR MICROENVIRONMENT

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Introduction. Multiple Myeloma (MM) is a clonal B-cell malignancy characterized by accumulation of malignant plasma cells (PCs) within the bone marrow (BM) in close contact with stromal cells (SCs) which secrete growth factors and cytokines, promoting tumor cell growth and survival. The rapid progression of MM is dependent upon cellular interactions within the BM microenvironment, and novel agents targeting this interaction appear to be promising therapeutic strategies for the treatment of MM tumor expansion. It is well established that hypoxia is an important selective force in the evolution of tumor cells and a stabilization of HIF-1α protein has been documented in several human cancers. In MM, HIF-1α expression has been described in several cell lines and in about 35% of CD138+ cells isolated from MM patients samples, suggesting an oxygen independent stabilization of the protein. *Methods.* Given the importance of the BM microenvironment in MM pathogenesis, we evaluated the effect of permanent suppression of HIF-1α in MM cells-BMSCs communication by using a 3rd generation antisense oligonucleotide, EZN-2968. *Results.* Firstly, we confirmed that EZN-2968 down-regulated HIF-1α mRNA in all MM cell lines tested when cultured alone or in combination with BMSCs. Although we did not find a significant inhibitory *in vitro* effect on MM cell proliferation and survival by HIF-1α suppression as we previously reported, here we showed that upon exposure to HIF-1α inhibitor, neither the incubation with IL-6 nor the co-culture with BMSCs were able to revert the anti-proliferative effect induced by exposure to EZN-2968. Moreover, we also confirmed a significant reduction of IL-6 and VEGF in the media culture upon treatment with EZN-2968 alone or in combination with BMSCs, thus supporting the involvement of HIF pathway in the regulation of IL-6 and VEGF secretion. Gene expression profile analysis of MM cells after treatment with EZN-2968, showed an overall down-modulation of the VEGF family signaling pathway, supporting our previous findings. We further characterized the effect of HIF stable inhibition on MAPK phosphorylation in MM cells. We observed that EZN-2968 down-modulates cytokine induced signaling cascades after a short incubation with BMSCs, and seems to induce a negative modulation of those transcripts previously shown to reflect the activation state of spe-

cific tumor cell pathways. This observation was also supported by gene expression profile experiments. We next evaluated the adhesion of MM cells to the extracellular matrix protein (ECM) in the presence of stroma-derived supernatant. One of the key findings of our study is that PC attachment to the ECM was reduced in MM cells incubated with or without BMSCs in the presence of EZN-2968. *Conclusions.* Overall, these results strongly support the concept that HIF-1α plays a critical role in the interactions between BMSCs and PCs. We conclude that HIF inhibition may be an attractive therapeutic target for MM.

C021

LIGHT INVOLVEMENT IN THE IMPAIRED OSTEOBLASTOGENESIS OCCURRING IN MULTIPLE MYELOMA-BONE DISEASE

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Introduction. Multiple myeloma (MM)-bone disease is characterized by unbalanced bone remodelling, due to increased osteoclastogenesis and impaired osteoblast (OB) repair. Although largely studied, the osteoblastogenesis regulation in MM-bone disease needs to be further clarified. LIGHT/TNFSF14 is expressed by activated T-cells, monocytes, granulocytes, spleen and immature dendritic cells. Its expression was reported in rheumatoid arthritis patients with erosive bone disease; we purposed to investigate whether LIGHT is implicated in the mechanisms leading to the osteoblastogenesis impairment occurring in MM-bone disease. *Methods.* PB and BM aspirates were obtained from 40 patients (23M/17F, median age: 64 years), newly diagnosed as having symptomatic MM with or without bone disease, smoldering MM (sMM) or M.G.U.S. Bone disease assessment was performed by skeleton x-Ray, and spine and pelvis NMR or CT. The control group included PB and BM aspirates from 15 patients with non-neoplastic disease without any skeletal involvement, and PB from 25 healthy donors matching for age and sex with the MM group. Patients and controls gave their written informed consent to the study, approved by Ethical Committee of University Hospital of Bari, and performed according to Declaration of Helsinki. By means of flow cytometry, western blotting, and real-time PCR, LIGHT expression was assessed in freshly purified CD14+ monocytes, CD2+ T-cells and neutrophils from PB and BM aspirates of patients and controls. In cultures from BM mononuclear cells (BMNCs), the formation of CFU-F and CFU-OB was evaluated in the presence or absence of anti-LIGHT neutralizing mAb. CFU-F and CFU-OB were identified with alkaline phosphatase (ALP) or Von Kossa staining, respectively. Further, in CFU-F and CFU-OB cultures, the expression of OB differentiation markers was analyzed by real-time PCR. *Results.* In CD14+ monocytes, CD2+ T-cells and neutrophils from PB and BM of patients with MM-bone disease, we detected at both protein and mRNA levels a LIGHT higher expression than in the cells from patients with symptomatic MM without bone disease, sMM, M.G.U.S, non-neoplastic disease, and in healthy donors. The *in vitro* effect of anti-LIGHT mAb on osteoblastogenesis resulted in a significantly dose-dependent increase of the CFU-F and CFU-OB formation ($p<0.001$). In CFU-F cultures from MM-bone disease patients, we found a low expression of ALP and collagen-I, strongly increasing after addition of anti-LIGHT mAb. In untreated CFU-OB cultures, we detected a low expression of bone-sialoprotein-II and osteocalcin (matrix glycoproteins, typically expressed during the late phase of OB differentiation) as well as of Fra-2 and Osterix (transcription factors). Significantly increased mRNA levels of the above molecules were detected in the presence of anti-LIGHT mAb ($p<0.001$). *Conclusions.* These findings suggest a LIGHT involvement in the osteoblastogenesis impairment occurring in MM-bone disease.

C022

ACTIVATION OF THE MTOR PATHWAY AND LOCALIZATION OF THE mTOR PROTEIN IN PRIMARY MYELOMA CELLS AND MULTIPLE MYELOMA CELL LINES: ROLE OF POMALIDOMIDE

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Introduction. mTOR is a protein kinase that plays a central role in regulating critical cellular processes. Aim of this work is to evaluate 1) activation of the mTOR pathway in Multiple Myeloma (MM); 2) cellular localization of mTOR protein in MM cell lines and primary MM cells; 3) the role of pomalidomide in regulating mTOR. *Methods.* Immunohistochemistry with p-mTOR, p-AKT, p-P70S6K and p-4EBP-1 were performed on bone marrow sections of MM patients. Following pomalidomide incubation, proliferation and apoptosis were evaluated on MM cell lines and in primary myeloma cells. Cellular localization of mTOR was defined with confocal microscopy in basal condition and after pomalidomide treatment. Cytoplasmic and nuclear fractions were analysed by western blotting with mTOR, p-mTOR, AKT, p-AKT in MM cell lines after pomalidomide incubation. *Results.* Cytoplasmic p-mTOR stained positive in 57 out 101 (57.6%) cases by immunohistochemistry with a nuclear p-mTOR localization in 14 out 101 cases (13.8%). In the 70 MM patients evaluated for the entire pathway, p-mTOR expression significantly correlated with p-AKT ($r=0.29$, $P=0.05$), p-P70S6K ($r=0.35$, $P=0.001$), and p-4E-BP1 ($r=0.41$, $P=0.0001$). p-mTOR positive staining (HSCORE ≥30) significantly correlated with high LDH serum levels ($P=0.003$) while strong p-mTOR expression (HSCORE ≥80) significantly correlated with β2-microglobulin serum level higher than 5.5 mg/L ($P=0.023$) in the 57 newly diagnosed MM patients. Pomalidomide 1μM at 48h significantly suppressed proliferation of OPM-2 and RPMI8226 cells with IC50 values of 0.25 μM and 0.6 μM, respectively. Pomalidomide 1μM was also effective in plasmacells from 3 MM patients at 24h with 23%, 33% and 26% annexin-V positive cells. Immunofluorescence assays demonstrated that mTOR protein is distributed throughout the cytoplasm and the nucleus at baseline in MM cell lines and in plasmacells of 3 out 4 MM patients and a clearly increase of nuclear mTOR protein was detected in the some samples following pomalidomide incubation. Moreover, mTOR and nucleolin-co-localization was evidenced in RPMI8226, OPM2 cells and in plasmacells from 9 MM patients. Cytoplasmic and nuclear distribution of mTOR and p-mTOR was also evidenced by western blotting in RPMI-8226 and OPM-2 cells. As expected, the mTOR and p-mTOR protein levels were significantly higher in the cytoplasm when compared to the nucleus. Treatment with pomalidomide 10μM at 48h increased nuclear mTOR and p-mTOR expression levels in the nucleus. *Conclusions.* 1) AKT/mTOR pathway is activated in a subset of MM patients; 2) in MM cell lines and in a fraction of primary MM cells, mTOR is distributed throughout the cell cytoplasm and in some nucleus; 3) the anti-myeloma activity of pomalidomide may be mediated by the downregulation of the mTOR pathway with a nuclear shuttling of mTOR and p-mTOR protein.

C023

ROLE OF PD-1/PD-L1 AXIS IN THE IMMUNE PARESIS OF BONE MARROW Vγ9Vδ2 T CELLS IN MULTIPLE MYELOMA

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Introduction. Multiple Myeloma (MM) is a paradigm disease where tumor cells proliferate, resist to chemotherapy and escape immune surveillance by interplaying with the surrounding microenvironment. We have previously shown that approximately 50% of MM patients have peripheral blood (PB) Vγ9Vδ2 (γδ) T cells refractory to proliferation induced by isopentenylpyrophosphate (IPP) generated by zoledronic acid (ZA)-treated monocytes [non-responders (NR)]. However, we have previously demonstrated that NR status of PB γδ T cells can be reverted using ZA-treated DC (DCZA+) rather than monocytes as IPP producers. Recently we observed that γδ T cells from bone marrow (BM)

of MM patients have lost their immune competence and become anergic to ZA-stimulation. The aim of this work was to identify strategies to overcome myeloma-induced BM γδ T-cell immune dysfunction and restore their anti-myeloma activity. *Methods.* γδ T cell immunophenotype: four color flow cytometry was used to determine the subset distribution of γδ T cells, the expression of the inhibitory receptors (PD-1 and BTLA) and of its ligands (PD-L1 and HVEM) in the tumor microenvironment. γδ T cell activation: MM BMMC were cultured for 7 days in presence of IL-2 and ZA. On day 7, percentages and total counts of viable γδ T cells were calculated with the trypan blue staining and flow cytometry. BM γδ T cell proliferation and CD107 expression was also evaluated in the presence of anti-PD1 blocking mAb. Myeloid-derived suppressor cells (MDSC) and regulatory T cells (Tregs) inhibition: to test the role of MDSC and Tregs in the anergy of MM BM γδ T cell, BMMC were cultured for 7 days with IL2 and ZA in presence or absence of functional inhibitors of these suppressor cells (sildenafil and 1-methyl-tryptophan for MDSC;OX40L and anti-TGFβ for Tregs). *Results.* Our results point to a functional exhaustion of BM γδ T cells driven by the local microenvironment as the main cause of immunoparesis. Tumor cells and bystander cells in the BM microenvironment of MM patients have an extremely accelerated Mevalonate pathway activity leading to high concentrations of extra-cellular IPP in the tumor site. We investigated the expression of programmed death-1 (PD-1) receptor, a negative regulator of T cell activation and proliferation, on BM and PB γδ T cells from MM patients and healthy donors. Interestingly, MM BM γδ T cells showed the highest PD-1 expression, mainly in the subset with the highest proliferative capacity (central memory). Moreover, our data indicate that the late removal of Tregs and MDSC, negative regulators locally recruited by myeloma cells, is not sufficient to reinstate the immune competence of BM γδ T cell. Besides, our results reveal that BM γδ T cells dysfunction is an early and long-lasting event during the disease evolution. *Conclusions.* Our results evidence that neutralization of the PD-1/PDL-1 axis partially reinstates BM γδ T cell reactivity and improves their cytotoxic ability in MM patients.

C024

THE ROLE OF NOTCH PATHWAY IN MULTIPLE MYELOMA PROGRESSION TOWARD IL-6 INDEPENDENCE

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Introduction. Multiple myeloma (MM) is a heamotologic malignancy characterized by proliferation of neoplastic plasma cells in the bone marrow (BM). Initially, myeloma cells strictly depend on BM, which supports tumor progression through adhesion molecules and soluble mediators as interleukin-6 (IL-6). Later, at the stage of plasma cell leukemia, MM cells acquire mutations resulting in proliferation indipendent from environmental factors such as IL-6. Recently, Notch signaling has been shown to be upregulated during MM progression and to positively regulate cell proliferation, drug resistance and BM infiltration. The aim of this study is to evaluate if Notch signalling plays a role in the acquirement of IL-6 independence. *Methods.* The human MM cell lines CMA03, INA-6 and XG1 were maintained in RPMI-1640 medium supplemented with IL-6 (respectivetly at 10, 2.5 and 1ng/ml). CMA03/06, OPM2 and U266 were maintened in the same condition withouth IL-6. The murine fibroblasts NIH3T3 were used as BMSC mimic and maintained in DMEM. Viable cells were counted by trypan blue exclusion assay. Notch inhibition was obtained by using the γ-secretase inhibitor DAPT at 50mM, soluble Jagged1 was used at 5mg/ml. qRT-PCR reactions were performed by Maxima™ SYBR GreenqPCR Master Mix. Silencing of Jagged1 and 2 was obtained by transient expression of specific siRNAs (Select RNAiTM siRNA system, Invitrogen). *Results.* The global expression analysis of the MM model of IL-6 independence acquisistion represented by CMA03 and CMA03/06 cell line (Verdelli *et al.* Genes Chromosomes Cancer, 2014), indicated that Notch pathway activation may contribute to the IL-6 independence in MM by inducing proliferative signals. Accordingly, we showed here that the activation of Notch signaling, induced by stimulation with soluble Jagged1 ligand, partially rescued IL-6 dependency in XG1 cells. Otherwise, Notch signaling inhibition obtained with DAPT in three different IL-6-indipendent MM cell lines (CMA03/06, OPM2 and U266) resulted in a significant decrease of cell growth which could be reverted by IL-6. This confirms that Notch and IL-6 are complementary in activating MM cell proliferation. Of note, Notch withdrawal induced by Jagged1/2 silencing, decreased IL-6 expression in OPM2 and U266 cell lines. This suggests that Notch-directed IL-6 regulation might have a biological significance in those MM cell lines which express high IL-6 levels. More frequently, BM stromal cells represent the main source of IL-6 for those MM cells which do not display an autonomous production. Results from co-culture systems indicate that surface Jagged expressed on MM cell lines induced Notch-directed IL-6 production in stromal cells. This effect was reverted by silencing Jagged1/2 in MM cells. *Conclusions.* These results suggest that Notch pathway activation may contribute to the transition from IL-6-dependent to IL-6-independent cell growth, and that its inhibition may result in decreased cell proliferation.

Stem Cell Transplantation and Cell Therapy

C025

CRUCIAL FACTORS OF THE INFLAMMATORY MICROENVIRONMENT ABNORMALLY MODULATE SURVIVAL AND PROLIFERATION OF CIRCULATING CD34+ STEM/PROGENITOR CELLS OF MYELOFIBROSIS

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Introduction. Myelofibrosis (MF) is an acquired clonal disorder of the hematopoietic stem/progenitor cell (HSPC). Mutations in JAK2 or Calreticulin genes are associated with MF (around 70-80% of cases). MF is characterized by a state of chronic inflammation and it is argued that the up-regulated production of proinflammatory cytokines by both HSPCs and the surrounding stromal cells generates a microenvironment that selects for the malignant clone. However, the key players linking inflammation and cancer in MF as well as the underlying pathogenetic mechanism are still to be defined. Extracellular nucleotides and selected cytokines, such as Tumor Necrosis Factor (TNF)-alpha or the Tissue Inhibitor of Metalloproteinases-1 (TIMP-1), are crucial factors of the inflammatory microenvironment. Here we investigated the *in vitro* effects of ATP, TNF-alpha or TIMP-1 on phenotype/function of HSPCs from MF patients. *Methods.* Circulating CD34+ HSPCs from MF patients with JAK2V617F mutation (7 cases) or cord blood (5 samples) have been phenotypically and functionally characterized at baseline or after *in vitro* incubation for 24 hours with or without increasing doses of ATP, TNF-alpha or TIMP-1. Phenotype: the following markers have been analyzed by flow cytometry: CD133, CD45, CD184, CD105, CD117, KDR, CD41, CD63, CD44, CD49D. Apoptosis assay: immunomagnetically isolated CD34+ cells were stained with Annexin-V/Propidium Iodide and analyzed by flow cytometry. Migration assay: purified CD34+ cells were assayed towards a CXCL12 gradient (150ng/mL) in transwell chambers. Clonogenic Assay: purified CD34+ cells were *in vitro* incubated in methylcellulose medium. *Results.* We found that circulating CD34+ cells from MF patients show reduced expression of CD63 (TIMP-1 receptor). Accordingly, the clonogenic expansion of MF CD34+ cells after *in vitro* incubation with TIMP-1 (100 ng/mL) was lower than that of the cord blood-derived CD34+ HSPCs (0,98 *vs* 1,7 fold increase as compared with untreated samples, respectively). We also found that survival of MF CD34+ cells was strongly stimulated by TNF-alpha (10 ng/mL; 2,9 fold increase) as compared with that of cord blood-derived CD34+ cells (1,7 fold increase). Accordingly, TIMP-1 treatment promotes the survival of MF CD34+ cells. However, at variance with cord blood-derived CD34+ cells, the MF CD34+ cells were more resistant to the citotoxic activity of high dose ATP (1nM). In addition, despite CXCR4 expression of CD34+ cells was not significantly different between patients and controls, when we analyzed the *in vitro* migration of CD34+ cells toward a CXCL12 gradient, we observed a slightly reduced migration capacity of MF CD34+ cells as compared with the normal counterparts. *Conclusions.* Survival and proliferation of circulating CD34+ HSPCs of MF are abnormally modulated by selected inflammatory factors. This finding may contribute to clarify the role of the inflammatory microenvironment in the pathogenesis of MF.

C026

A NOVEL MULTIPARAMETER RISK SCORE OF CHRONIC GVHD BASED ON THE MEASUREMENT OF IP10 (CXCL10) AND PLASMACYTOID DC (pDC) IN THE PERIPHERAL BLOOD AT 3 MONTHS AFTERTRANSPLANT

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Introduction. Chronic GVHD (cGVHD) is the major cause of long term morbidity after allogeneic HSC transplantation. No biomarkers are currently known that can consistently predict its occurrence. We have previously observed that patients with cGVHD have increased numbers of circulating activated monocytes (Arpinati Transplantation 85:1826; 2008). Also, the serum concentration of inflammatory chemokines has been associated with acute GVHD (aGVHD). We thus aimed to evaluate whether PB numbers of antigen-presenting cell (APC) subsets or serum chemokine concentrations are biomarkers of chronic GVHD occurrence. *Methods.* PB samples were collected at 3 months after transplant. 102 consecutive patients undergoing transplant between 2007 and 2011 were included in the study, provided they were evaluable for chronic GVHD. Multicolor flow cytometry was employed to determine the number of PB myeloid (mDC) and plasmacytoid DC (pDC), CD16+ DC, and CD16+ and – monocytes, as well as CD4+ and CD8+ T, CD56+ NK and CD19+ B cells. The serum concentration of IL-8, IP-10, MCP1, MIP-1alpha and –beta and RANTES was measured by cytometry bead array (CBA) assay. *Results.* After a median of 72 days (IQ 27-174) following enrollment, 40 patients had developed cGVHD (25 extensive) with an actuarial probability of 51%. Patients with and without cGVHD had comparable clinical characteristics. However, more patients with cGVHD had had previous aGVHD (24/40, 14 grade II-IV, *vs* 18/62, 8 II-IV) ($p<0.01$). ROC analyses were performed to screen each potential biomarker for its ability to discriminate patients developing cGVHD. A significant correlation with cGVHD (based on the area under ROC curve >0.5) was found for IL-8, IP10, MIP1-a, MIP1-b, mDC, pDC, CD3 and CD8 levels in the PB. A multivariate Spearman correlation analysis identified the following variables as independently associated with the risk of cGVHD: IP10 > 557 pg/ml (OR 4.9, CI 1.7-13), pDC < 2.3 per mcl (OR 5.3, CI 1.7-17) and previous aGVHD (OR 2.7 CI 1.4-4.9). A risk score was developed based on the weighted coefficients of each variable (3 points for IP10 and pDC, 2 for previous aGVHD). 10/49 patients (20%) with a score of <2, 14/29 (48%) with a score between 3 and 5 and 14/17 (82%) with a score >6 developed cGVHD ($p=0.0002$). The AUC of the ROC curve of the risk score was highly significant (0.821, CI 0.72-0.89; $p<0.0001$). *Discussion.* A multiparameter score including the concentration of the chemokine IP10 and the number of circulating pDC at 3 months after transplant may stratify patients at low, intermediate or high risk of chronic GVHD.

C027

IMPACT OF CD3/T REGS RATIO IN DONOR GRAFT ON 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 post allotransplant patients' outcomes, there is no evidence that donor graft CD3/T regs ratio may determine an effect in terms of OS, NRM and relapse free survival rates so far. In this study we analyzed the graft CD3+/Tregs ratio (gCD3/Tregs R) and determined its impact on acute GVHD (aGVHD) and survival rates (OS, NRM and Relapse) after myeloablative alloPBSCT. *Methods.* We analyzed 102 consecutive patients (median age 39 yy) transplanted with unmanipulated peripheral blood stem cells from an HLA identical related donor (n=78) or an HLA identical unrelated donor (n=34); diagnoses were acute myeloid leukaemia (n=82), acute lymphoblastic leukaemia (n=20). *Results.* The median CD3+ and Tregs dose administered was 240 (range (r): 67-550) and $13x10^6$/Kg (r: 2-21), respectively; the median gCD3/Tregs R was 22 (r: 8-250). The median CD3+ and Tregs dose administered was 240 (range (r): 67-550) and $13x10^6$/Kg (r: 2-21), respectively; the median gCD3/Tregs R was 22 (r: 8-250). Patients were subdivided into a high gCD3/Tregs R(>36)group(n=46) and a low gCD3/Tregs R(<36) group (n=56). 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 (10/56 or 18% *vs* 35/46 or 77%, $p<.001$). The OS, NRM and relapse rate at 3 years was 54, 29 and 34%, respec-

tively. Comparing LR with HR group a statistically significant difference is demonstrated for OS and NRM rates at 3 years (65 *vs* 31%,p<.004; 3 *vs* 71%, p<.001), respectively, but not for the R one (35 *vs* 30%, p=ns; table 1). Comparing aGVHD+ with aGVHD- group OS, NRM and relapse were always statistically significant different at 3 years (39 *vs* 65%,p<.005; 61 *vs* 7%,p<.001; 9 *vs* 53%,p<.002). Conclusions. Taken together, our data may suggest that Tregs content is able to mediate protective effects against aGVHD, while preserving GvL effects as demonstrated by relapse rate comparison between H and LR groups. However, larger studies are needed to understand the real contribution of gCD3/Tregs R on survival rates.

C028

PLATELET LYSATE ENHANCES THE THERAPEUTIC ACTIVITY OF ADIPOSE DERIVED MESENCHYMAL STROMAL CELLS ISOLATED FROM CROHN DISEASE PATIENTS IN A NOVEL MOUSE MODEL OF COLITIS

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Introduction. Crohn's disease (CD) is a chronic inflammatory autoimmune bowel disease (IBD) characterized by segmental transmural inflammation. In 1/3 of patients, CD is complicated by perianal fistulas which rarely heal spontaneously or after medical treatment. Disappointing results have been obtained in anti-inflammatory drug-based (TNF-inhibitors) clinical trials so that 2/3 of patients either do not respond or loose their response. Pre-clinical studies and preliminary clinical trials have suggested that, due to their immunomodulatory properties, mesenchymal stromal cell (MSC)-based therapy hold promising potential in the IBD treatment. Ongoing studies are aimed at evaluating the proper dose and mode of MSC administration needed for more effective results. Human platelet lysate (HPL) has been proposed as a valid FBS substitute to safely expand MSCs for therapeutic purpose. A recent paper demonstrates that in MSCs HPL triggers in the secretion of factors enhancing the initial inflammatory response to the injury, a key element in the wound healing process. We recently set up a novel murine model of IBD. This model, conversely to that adopted so far, does not lead to animal death, but it presents a mild intestinal damage which makes it the most accurate to study therapeutic agent effects. The aim of our study was to characterize the effects of MSCs isolated from adipose tissue of CD patients and resuspended in HPL (adCD-MSCs/HPL) in our IBD model. *Methods.*Colitis was induced in C57BL/6J mice by administration of 1,5% dextran sulphate sodium (DSS) in tap water. adCD-MSCs, w/wo HPL, were administrated via enema for 3 times ($1x10^6$ cells/mouse/time) every other day starting on day+7 from DSS induction. *Results.*We found that adCD-MSCs can be easily isolated and expanded from CD patients, showing typical MSC properties. We demonstrated that HPL potentiated the adCD-MSC efficacy in ameliorating DSS-induced colitis in our model. Indeed, DSS+adCD-MSC/HPL-treated mice group showed a reduced weight loss compared to DSS+PBS mice group. Disease Activity Index, including inflammation clinical parameters (weight loss, diarrohea, rectal bleeding), was lower in DSS+adCD-MSC/HPL-treated mice at each time point starting from the end of MSC treatment. Histopathological examination of the colon distal and proximal tracts showed that adCD-MSCs/HPL significantly reduced the extension and the severity of the inflamed area and the inflammatory cells infiltration due to DSS treatment. We also found that adCDMSCs/HLP decreased systemic inflammatory mediators levels (IL-1β, IL-6, IL-17, IFN-γ, TNF-α, IL-10) in the colitic mice plasma. Finally, we set up a novel method to label adCD-MSCs. We found that Nile red staining was very efficient and stable and preserved MSC biological properties. Thus, Nile red staining can be efficiently used to track injected MSCs. *Conclusions.*Overall, this study validate a novel promising adCD-MSC/HPL-based therapy for refractory CD treatment.

C029

HIGH DOSE THERAPY WITH BENDAMUSTINE, ETOPOSIDE, ARA-C, MELPHALAN (BEEAM) PRIOR TO AUTOLOGOUS STEM CELL TRANSPLANT IS SAFE AND EFFECTIVE IN AGGRESSIVE B-CELL NON HODGKIN LYMPHOMA: AN ITALIAN PHASE II, MULTICENTER STUDY

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Introduction. We previously demonstrated (Visani *et al.,* Blood 2011) the safety of a new conditioning regimen with bendamustine, etoposide, cytarabine, and melphalan (BeEAM) prior to autologous stem cell transplant (ASCT) in resistant/relapsed lymphoma patients (EUDRACTnumber2008-002736-15). The regimen showed long-lasting significant anti-lymphoma activity, with a 3-year PFS of 75%. *Aims.* We designed a phase II study to confirm the effectiveness of BeEAM as a preparative regimen for autologous stem cell transplantation in resistant/relapsed aggressive B-cell non-Hodgkin lymphoma patients. The study was registered at EMEA with the EUDRACT no 2011-001246-14.The primary end-point of the study is to evaluate the 1-year complete remission rate. Fixing the lowest acceptable rate as 55% and the successful rate as 70%, with a significance level alpha=0.05 and a power 1-beta =0.90, the sample size was estimated in 88 patients. *Methods.* until now, 37 patients (median age 56 years, range 19-69) with resistant/relapsed diffuse large B cell (32) or grade III B follicular (5) non-Hodgkin lymphoma were consecutively enrolled in the study. Briefly, 27 patients had advanced stage disease (III-IV), 12 were primary refractory and 25 had relapsed after a median number of 2 lines of therapy (range: 2-3). Thirty-three patients had good performance status (WHO 0-1), and 11 patients presented with 1 or more relevant comorbidities (range: 1-5). Nineteen patients were in II or subsequent CR after salvage therapy, whereas 16 were in PR and 2 had progressive disease. *Results.* A median number of 5.84x10e(6)CD34+/kg cells (range 2.8-8.8) collected from peripheral blood was reinfused to patients. All patients engrafted, with a median time to ANC>0.5x109/l of 10 days. Median times to achieve a platelet count >20x10e(9)/l and >50x10e(9)/l were 12 and 16 days respectively. Eight out of 37 patients presented a fever of unknown origin (21.6%), whereas 19 patients (51%) presented a clinically documented infection. All patients received G-CSF after transplant for a median time of 8 days (range: 8-13). One patient died due to an incomplete hematological recovery after transplant, producing an overall transplant related mortality of 2.7%. Twenty-seven out of 37 patients are evaluable up to now for response to treatment. 22/27 (81.5%) obtained a CR, 2/27 a PR, whereas 3/27 did not respond to therapy. After a median follow-up of 9 months from transplant (range 2-24), 5/24 patients relapsed, whereas 19/24 (79.1%) are still alive, in continuous CR. *Conclusions.* The BeEAM regimen preliminary confirmed its safety (TRM 2.7%) and its promising efficacy in resistant-relapsed aggressive B-cell lymphomas. *Acknowledgements.* We kindly acknowledge Mundipharma Europe and Mundipharma Italy for providing the drug for the study. The study was supported in part by AIL Pesaro Onlus.

C030

FEASIBILITY AND EFFICACY OF A NEW SCHEDULE OF MOBILIZATION WITH BORTEZOMIB AND CYCLOPHOSPHAMIDE IN 61 MULTIPLE MYELOMA PATIENTS: A PROSPECTIVE STUDY

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Introduction. Autologous Stem Cell Transplantation (ASCT) is a mainstream therapy in Multiple Myeloma (MM); however, in a relevant proportion of patients (pts) the optimal target of peripheral blood stem cells (PBSCs), cannot be achieved. The two most commonly used mobilization regimens are the cytokine granulocyte colony-stimulating factor (G-CSF) alone at 10 mcg/kg/day or intermediate-dose Cytoxan (CTX) followed by G-CSF. The latter is still the most widespread schedule for MM mobilization in Italy and it has been reported to be associated with better harvest, even though it does not seem to reduce the percentage of mobilization failures. In the "new drugs era", the potential anti myeloma activity of CTX and the real utility of this drug, both for improving the mobilization outcome and for reducing the harvest contamination, is still debated. We report the results of a new CTX/Bortezomib/G-CSF-based mobilization schedule in a prospective multicenter study, in fit elderly pts (> 60 years) with MM, candidate for ASCT. *Methods.* We enrolled 61 pts (31 F, 30 M), with a median age of 64 years (range: 60-78), who received a first line therapy with Bortezomib, Cyclophosphamide and Dexamethasone (BOR 1.3 mg/m2/dose on days 1-4-8-11, i.v. DEX 40 mg/day on days 1-4-8-11 and i.v. CY 300 mg/m2/dose on days 1-8-15) for at least 4 courses; pts achieving at least a PR after 4 courses of Cy-BOR, proceeded to mobilization; mobilization schedule consisted in: i.v. BOR 1.3 mg/m2/dose (days 1-4-8-11), i.v. DEX 40 mg/day (days 1-4-8-11) and i.v. CY 3000 mg/m2/dose (day 8). G-CSF, at 5mg/Kg/day, was administered starting from day 9. *Results.* 53 pts are evaluable for response after induction; 41 achieved ≥PR (77%); 39 pts underwent mobilization (two pts withdraw the consent) and 38 pts (97%) collected ≥2.0×10^6 CD34+ cells/Kg (median: 6.4 $\times10^6$ CD34+/Kg, range: 2.75-15.8) in a median number of two aphereses (range1-4). 33 pts (86%) collected ≥4.0×10^6 CD34+/Kg. This schedule was well tolerated: only one patient did not complete the mobilization procedure, due to a serious adverse event (dyspnea and pleural effusion due to progressive disease). We did not observe neither any grade cytopenia nor neutropenic fever. However we did not observe any response improvement after this schedule in any pts. "On demand" Plerixafor was administered to 5 pts. Evaluation of MRD in 20 leukaphereses by multiparametric flow cytometry showed a median plasma cells value of 0,03% (0,00-0,52%); in 6 pts PCs were < 0,01% (cut off for MRD negativity). *Conclusions.* in this subset of elderly MM pts the mobilization with bortezomib and CTX, followed by G-CSF is safe and effective. This schedule allows the collection of an adequate number of CD34+ stem cells, with a very low rate of mobilization failure (3%), also in elderly MM pts. However this approach does not seem to improve the response rate, although a relevant percentage (30%) of pts achieved MRD negativity in the harvest.

C031

OPTIMAL TIME-POINTS FOR MINIMAL RESIDUAL DISEASE MONITORING CHANGE ON THE BASIS OF THE METHOD USED IN PATIENTS WITH ACUTE MYELOID LEUKEMIA WHO UNDERWENT ALLOGENEIC STEM CELL TRANSPLANTATION: A COMPARISON BETWEEN MULTIPARAMETER FLOW CYTOMETRY AND WILMS' TUMOR 1 EXPRESSION

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Introduction. Allogeneic stem cell transplantation (allo-SCT) represents the only effective therapy for high risk patients with acute myeloid leukemia (AML). Nevertheless, relapse remains a crucial issue in this setting and new methods able to prevent it are needed. Monitoring of minimal residual disease (MRD) allows stratifying the patients on the basis of the risk to experience a recurrence, although an agreement on most sensitive method and timing are missing yet. Thus, we studied technical performance and prognostic impact of MRD assessed at the optimal cut-off by multiparameter flow cytometry (MFC) and WT1 expression, before and after transplant. Moreover, a comparison among positive, negative MRD patients and poor responders was further investigated before transplant. *Methods.* Between June 2010 and January 2014, 42 adult AML patients underwent allo-SCT at the Hematology Department of IRCCS "Casa Sollievo della Sofferenza" Hospital. Samples for MRD analysis were collected before (-10 days) and after transplant (+ 30 days) and were investigated by 6-color MFC and quantitative analysis of WT1. All patients received a myeloablative conditioning regimen. Patients showing partial remission (PR) or refractory disease at the time of transplant were defined as poor responders. *Results.* When both methods were compared at the optimal cut offs before transplant, MRD identified by MFC showed better values of sensitivity (71.4% *vs* 57.1%), specificity (87.0% *vs* 72.7%), positive predictive value (PPV) and negative predictive value (NPV) than WT1. After transplant, MFC displayed a slightly higher sensitivity (80.0% *vs* 75.0%) but lower value of specificity (65.5% *vs* 88.5%), positive predictive value (PPV) (80.0% *vs* 59.3%) and NPV compared to WT1. Patients with pre-transplant MRD values greater than 0.1% by MFC showed a higher risk of recurrence compared to others with lower values (p=0.006). Only a trend to significance was found on DFS between patients having more and less than 64 copies WT1/104 ABL (p=0.081). Interestingly, MRD positive patients showed a worse DFS compared to poor responders, although differences were not significant (p=0.465), while negative patients had a better DFS compared to both positive patients (p=0.003) and poor responders (p=0.031). Post-transplant threshold of 0.04% by MFC identified patients with a poorer prognosis in terms of both DFS (p=0.013) and OS (p=0.034). Similarly, a shorter DFS was found in patients with levels above 125.0 copies WT1/104 ABL by WT1 expression (p=0.001). At multivariate analysis were confirmed results obtained at the univariate. *Conclusions*. In conclusion, our results support the idea that MRD monitoring by MFC should be preferred before the transplant and WT1 expression after. The negative impact on prognosis of pre- and post- transplant positive MRD as well as poorer outcomes of MRD positive patients compared to poor responders define MRD monitoring as mandatory in this setting.

C032

DEFERASIROX AND ROS PRODUCTION IN HEMATOPOIETIC STEM CELLS: A NEW MOLECULAR MECHANISM FOR MYELODYSPLASTIC SYNDROMES TREATMENT

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Introduction. Deferasirox (DFX) is an iron chelator used to prevent and treat complications related to transfusional iron overload in myelodisplastic syndrome patients (MDS). Intriguingly, DFX treatment induces haematological responses in a consistent percentage of patients whereas other chelators, like deferoxamine (DFO) do not show such an appreciable effect. Consolidated evidence highlights the importance of redox signalling in the homeostasis of fundamental processes, particularly in controlling the balance between self-renewal and differentiation of stem cells. In this setting, reactive oxygen species (ROS) would act as secondary messengers, modulating the expression of master transcrip-

tion factors and regulatory proteins leading or (pre)conditioning stem cells towards differentiation. In the present study we investigated the effect of DFX on ROS production in hematopoietic stem cells (HSCs) in order to identify a molecular mechanism explaining the differential effect of iron chelators in rescuing altered hematopoiesis. *Methods.* Human HSCs, isolated upon informed consent from peripheral blood of G-CSF treated healthy donors, were treated with 100μM DFX or DFO for 24 hours. To completely abrogate ROS production, cells were co-incubated with diphenil iodide (DPI) 100μM. ROS levels were analyzed by laser scanning confocal microscopy (LSCM) and flow cytometry after the incubation at 37°C for 15min with the intracellular H_2O_2 specific probe dichlorodihhyrofluorescein-diacetate (H2DCFDA)10μM. Oct4, sox2 and sox17 transcript levels were measured by real time PCR. β-catenin and BMI1 protein levels were assessed by western blotting. CD34 and CD71 expression and MitoTraker Green staining were analyzed by flow cytometry. Data, expressed as mean±s.e.m. were compared by unpaired Student T-Test; a $p<0.05$ was considered significant. *Results.* DFX treatment of HSCs resulted in a significant up-regulation of ROS levels (p=.001) whereas no significant change was observed after DFO treatment. DFX incubation led to an activation of oct4, sox2 and sox17 gene expression and the reduction of β-catenin and BMI1, factors involved in self-renewal and differentiation processes. No significant variation was observed after DFO treatment. Interestingly, all these modulations, specifically induced by DFX, were reversed by ROS abrogation obtained by DPI treatment. These molecular events triggered by DFX were also accompanied by an up-regulation of CD71, marker of erythroid progenitors as well as an increase in mitochondrial mass, inversely correlated to CD34 expression. *Conclusions.* Our data would suggest a novel mechanism by which DFX treatment, through ROS signaling activation, influences key factors involved in self-renewal/differentiation of HSCs. In this scenario, the modulation of ROS, because of their ability to restore the hematopoietic function, could be taken in account as potential further pharmacological target in MDS treatment.

Leukemias and Myelodysplasias II

C033

GENETICS OF T-ALL WITH CMYC-TRANSLOCATIONS

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Introduction. CMYC is a key oncogene and an effective therapeutic target in murine models of T-ALL (Roderick JE, Blood 2014). In human T-ALL high CMYC expression is mainly caused by activation of NOTCH1 and post-transcriptional mechanisms mediated by PTEN. The present study focused on human T-ALL with genomic rearrangements of CMYC to delineate the genetic profile and clinical-hematological features of these leukemia subgroups. *Methods.* CMYC was studied in 64 adults and 132 children belonging to the AIEOP, GIMEMA, and UK clinical trials with LSI MYC Dual Color, Break Apart Rearrangement Probe (Vysis-Abbott) and homegrown G248P8135G5, RP11-367L7 and RP11-26E5 clones. Affymetrix HU133 Plus 2.0 arrays were used for whole transcriptome profiling (GEP) and Cytoscan HD Platform for SNPa. CI-FISH was performed as described (La Starza R, Leuk Res 2013). NOTCH1, FBW7, and PTEN were analysed by DHPLC (Transgenomic) and/or sanger sequencing (AB3500 Genetic Analyzer). *Results.* Reciprocal translocations of CMYC were detected in 12 cases and involved TCR loci in 6 cases and non-TCR loci in the other 6. The 8q24 breakpoints clustered at the telomeric region of CMYC in all cases with TCR translocations, while in non-TCR translocation breakpoints fell downstream (3 cases) or upstream (3 case) CMYC. CMYC-translocations always occurred in association with other changes (range: 2-10 additional abnormalities) behaving as secondary lesions. Associations with the TAL/LMO subgroup (Pearson Chi-square, P=0,018), trisomy 6 and trisomy 7 (Chi-square, $P<0,001$) were statistically significant. Other frequent concurrent changes were CDKN2A/B deletions (83,3%) and PTEN inactivation (50%). According to their secondary nature, CMYC-translocations were found in variable sized subclones (range: 8-62%) in 6 cases, suggesting a contribution to disease progression rather than to disease initiation. Interestingly, CMYC-positive clones appeared to be treatment-resistant and sustain disease relapse in 2 cases with available longitudinal studies. Gene expression profile (GEP) showed high CMYC expression in all cases with translocations. Considering the group of TAL/LMO positive cases, GEP showed patients with or without C-MYC translocations clustered separately (Figure 1D), with 148 genes differently expressed (77 up- and 71 down- regulated). A significant high expression of CD44 and downregulation of NOTCH1 was detected in CMYC-translocation positive cases. *Conclusions.* CMYC-translocations occurred in about 6% of T-ALL and were significantly associated with the TAL/LMO group and co-occurrence of poor prognostic markers, such as high CD44 expression and PTEN inactivation. Our cases recapitulated murine models of T-ALL in which c-Myc had a crucial role on maintenance and self-renewal of leukemia-initiating cells. Thus early identification, and eradication, of small CMYC-positive subclones at diagnosis and during treatment might be helpful to prevent disease progression.

C034

INHIBITION OF PROLIFERATION AND SURVIVAL IN T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA (T-ALL) CELLS REQUIRES BLOCKADE OF ALL THE CLASS I PI3Ks ISOFORMS

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Introduction. The heterodimeric class I phosphatidylinositol 3-kinases (PI3Ks) regulate a variety of cellular processes including cell proliferation, survival and metabolism, by generating the second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP3). PI3Ks are widely implicated in human cancers and in particular are upregulated in T-ALL, mainly due to the impairment of the lipid phosphatase PTEN function. At present different compounds which target single or multiple PI3K catalytic subunits have entered clinical trials. Here we have explored the role of the different PI3K isoforms in T-ALL in order to identify the most effective targeted therapy strategy. *Methods.* Both PTEN wt (ALL-SIL and DND-41) and PTEN deleted (Jurkat and Loucy) T-ALL cell lines were treated with class I pan-PI3K inhibitors (BKM120 and ZSTK454) or p110α (A-66), p110β (TGX-221), p110γ (AS-605240), p110δ (CAL-101) and p110γ/δ (IPI-145) selective inhibitors (Selleck Chemicals, Houston TX, USA), and their effects were evaluated. *Results.* In all the tested cell lines, flow cytometric analysis evidenced a reduction in PIP3 levels after all treatments, documenting PI3K inhibition. However, the extent of PIP3 reduction varied in the different cell lines and did not correlate to drug cytotoxicity. Indeed, only pan-PI3K inhibition significantly affected cell viability and proliferation in a dose- and time-dependent manner, with IC50 values ranging between 1 and 3.4 μM, for both BKM120 and ZSTK454, whereas for all the other drugs IC50 values were not attained. These findings suggested that antiproliferative effects are not exclusively dependent on PIP3 reduction. Moreover, only Loucy cell line resulted sensitive to the p110γ and γ/δ inhibitors, suggesting that a more complex cellular background might influence cytotoxicity. Consistent with these results, Annexin V/PI staining analysis showed an increase of apoptosis after 48h treatment in both PTEN wt and deleted cell lines only after treatment with pan-PI3K inhibitors, whereas cell cycle progression was not affected. PI3K signaling pathway analysis revealed the complete inactivation of the downstream Akt pathway almost exclusively after treatment with pan-PI3K inhibitors, as evidenced by decrease of p-Akt, both at Thr308 and Ser473, p-P70S6K and p-S6RP. Of note, p110γ and p110δ inhibition slightly affected these PI3K targets, but only in some cell lines. Furthermore, PI3K inhibition did not affect activity of PDK1 and PKC proteins. Overall, no differences emerged related to PTEN status. *Conclusions.* Here we demonstrated that, irrespective of PTEN status, only pan-class I PI3K inhibition is cytotoxic in T-ALL cells, implying that any isoform could sustain leukemic cell survival, and suggesting a redundant role played by each isoform. Therefore, our findings strongly support clinical application of pan-class I PI3K rather than single-isoform inhibitors in T-ALL treatment.

C035

GENETIC VARIANTS IN GENES INVOLVED IN FOLATE PATHWAY, DNA METHYLATION OR REPAIR PREDICT OVERALL SURVIVAL IN PATIENTS WITH LOW-RISK, UNTREATED MYELODYSPLASTIC SYNDROME

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Introduction. Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal hematopoietic disorders and are frequently associated with genetic instability. Genetic variants in DNA repair and folate-related genes have been correlated with the pathogenesis or progression of hematological malignancies. In this study we evaluated the impact of genetic variants in genes encoding main proteins of BER system (XRCC1, XRCC3 and APE1) and folate-metabolizing enzymes (TS, MTHFR) on survival of MDS patients with IPSS low-intermediate1. *Methods.* the study was designed according to the Schoenfeld design for biomarkers, assuming the presence of an unfavorable pharmacogenetic profile (one or more adverse genotypes) in at least one-third of the study population. Accordingly, 10 events in 54 patients would allow the detection of an Hazard Ratio (HR) >6 associated with the group having unfavorable genotypes (80% power and 5% type I error for a two-tailed test). Using PCR-HRM (High Resolution Melting) assay and restriction digests of PCR products, we prospectively studied the frequencies of the XRCC1 194 (rs1799782 C/T, Arg/Trp) and 399 (rs25487 G/A, Arg/Gln), XRCC3 241 (rs861539 C/T, Thr/Met), TS5'-UTR (2R/3R and rs183205964 G/C) and 3'-UTR (rs11280056 6bp+/6bp-), MTHFR 677 (rs1801133 C/T, Ala/Val) and 1298 (rs801131 A/C, Gln/Ala), APE1 148 (rs1130409 T/G, Asp/Glu) genotypes in 54 MDS patients (median age 75 years) with IPSS low (n=23) or intermediate-1 (n=31) receiving best supportive care. Genomic DNA was isolated from 1ml of peripheral blood by means of commercially available kits. The associations between genetic variants and survival were assessed using Kaplan-Meier method and Log-rank test. For the multivariate survival analysis, Cox proportional hazard models, was used to identify the genotypes. *Results.*. In multivariate analysis, a statistically significant adverse outcome was associated with XRCC1 399 GG, TS3'-UTR -6/-6, TS5'-UTR 2R/3G, 3C/3G, 3G/3G and MTHFR 677 TT variant alleles, with an Hazard Ratios of 4.65, 7.07, 11.44 and 67.12, respectively, if compared to the reference group of variant alleles (P=.058, P=.024, P=.026 and P=.000). Accordingly, we performed an exploratory analysis to investigate the effect on survival arising from the combination of the unfavorable genotypes to each other. Three-year OS was 33% for those patients with ≥2 variant alleles, as compared to 62.5%, and 100%, respectively, for those with 2 or 0/1 variant alleles, suggesting that patients with a higher number of genetic variants in genes involved in DNA methylation or repair and in folate pathway had a shorter survival. *Conclusions.* the genetic variants of genes involved in folate pathway, DNA synthesis or repair could predict prognosis and survival in low-risk MDS patients receiving best supportive care, if confirmed in larger series of patients. *Acknowledgements.* The study was supported in part by AIL Pesaro Onlus.

C036

MECHANISM OF RESISTANCE TO DECITABINE IN CHRONIC MYELOMONOCYTIC LEUKEMIA

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Introduction. In solid tumors, response to gemcitabine and other nucleoside analogues (NAs) is influenced by alterations of metabolizing enzymes. Recently, we demonstrated that in MDS patients the expression of UCK1, involved in the activation of azacitidine, may influence the clinical response to this treatment (Valencia *et al.,* Leukemia 2013). The NA 5-aza-2'-deoxycytidine (DAC) was demonstrated to have good efficacy in the therapy of chronic myelomonocytic leukemia (CMML). Given that no standard therapy has been identified so far for CMML, there is the need to identify molecular markers that could support the choice of DAC therapy. In the present study, we assessed the role of DAC metabolizing enzymes in the development of clinical resistance in CMML patients. *Materials and Methods.* Functional studies. The role of two main genes involved in DAC metabolism: DCK (DAC activa-

tion) and DCTD (DAC deactivation) was established using silencing by siRNAs in the MDS-SKM1 cell line treated with DAC 10uM for 48h. Patient samples. DNA and RNA were extracted from BM mononuclear cells of 38 CMML patients (16 defined as responders (R), 20 as non-responders (NR) to DAC, according to IWG 2006 criteria while 2 case were not-evaluable). Gene mutation. The fifteen most frequently mutated genes in CMML were sequenced at a mean depth of coverage of 520X(range 169–714X). Gene expression. RNAseq was performed in 14/38 CMML cases. cDNA libraries were sequenced using the HiSeq 2000. The counts of endogenous genes were normalized by ERCC spike-in library size, and the differential expression analysis was performed using edgeR (v3.4.2) GLM model. The differentially expressed genes were identified at the FDR cutoff of 0.05 and absolute fold change greater than 2. The expression level of genes involved in DAC metabolism (hENT1, hENT2, DCTD, hCNT3, CN-II, DCK and CDA) was also evaluated by Q-RT PCR. *Results.* In SKM-1 cells, DCTD silencing increased apoptosis (Annexin V-positive cells 20.2%±0.8% *vs* control 13.8%±0.5%; p=0.01). DCK silencing led to a decrease in apoptosis (Annexin V-positive cells 8.8%±0.1% *vs* control 13.8%±0.5%; p=0.05). The mutational frequencies (Figure 1) in this cohort of CMML cases were: 50% SRSF2, 39.4% ASXL1,18.4% NRAS, 44.7% TET2, 10.5% RUNX1 and DNMT3A, 7.8% U2AF1 and TP53, 5.2% KRAS, JAK2 and KIT, 2.6% EZH2, IDH1, IDH2 and SF3B1. No single genetic alteration was significantly associated with shorter overall survival or resistance to DAC. R and NR cases did not show a difference in expression levels of NA metabolizing enzymes by RNAseq. Q-RT PCR confirmed these observation specifically for the seven genes coding DAC metabolizing enzymes. *Conclusions.* CMML clinical resistance to DAC does not appear to be correlated with the expression of genes involved in DAC metabolism or to gene mutations. Further studies on larger series of CMML cases are needed to validate these results obtained in a small group of cases.

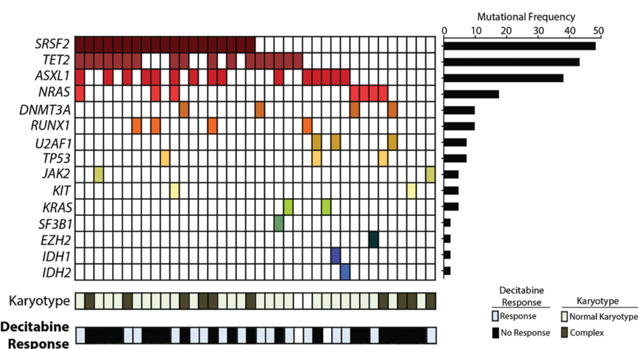

Figure 1.

C037

ROLE OF THE HYSTONE DEACETYLASE INHIBITOR GIVINOSTAT (ITF2357) IN TREATMENT OF CRLF2 REARRANGED ACUTE LYMPHOBLASTIC LEUKEMIA

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Introduction. Novel genomic abnormalities of CRLF2 and JAK2 genes have been recently reported in a subset of childhood ALL patients without known chromosomal aberrations, leading to the deregulation of CRLF2 cytokine receptor pathway and associated to a poor prognosis. Inhibition of CRLF2/JAK2 signalling has the potential to become a therapeutic intervention for this subgroup of patients. In addition to the use of JAK2 inhibitors, numerous reports indicate that a broader anti-tumor activity is necessary to effectively treat tumor cells with aberrant JAK2 signalling. Previous studies have shown that the HDAC inhibitor Givinostat/ITF2357 has potent anti-tumor activity against hematological malignancies, including myeloproliferative neoplasms (MPN) carrying the JAK2V617F mutation. Indeed, the molecule is currently being evaluated in a phase1/2 study in patients with polycytemia vera. In 10% of pediatric high-risk ALL and about 20% in Down Syndrome ALL patients, JAK2 mutations are characterized by a specific and frequent R683G mutation. Interestingly R683 and V617, commonly mutated in MPN, are located in the same JAK2 pseudokinase domain. This opens the possibility that Givinostat could have an inhibitory activity also on ALL blasts positive for JAK2 mutations and on ALL with disregulation of JAK/STAT pathway independently of JAK2 mutations. *Results.* Here we demonstrate that Givinostat at low concentrations inhibits proliferation and induces apoptosis in human B cell precursor leukaemia MHH-CALL4 and MUTZ 5 cell lines positive for JAK2 mutations (R683G and I682F respectively) and CRLF2 rearrangements. Givinostat inhibits proliferation with IC50 between 0.08-0.17 uM and induces apoptosis with IC50 between 0.17-0.25 uM. Moreover, at 100-200 nM, Givinostat downregulates the expression of JAK2, STAT5A and its target cMyc together with CRLF2 and IL7 receptor genes (RQ-PCR). Key factors of B cell development (EBF1, PAX5, IKZF1, E2A) are also downmodulated. Givinostat inhibits the phoshorilation of Stat5, a target protein downstream to CRLF2-mediated JAK-STAT pathway,both at a basal level and after stimulation with the CRLF2 ligand TSLP (phosphoflow analysis). Furthermore, low concentration (200 nM) of Givinostat inhibits proliferation and induces cell death of blasts from secondary xenograft with wt Jak2 and overexpressing CRLF2 due to P2RY8-CRLF2 fusion, at a three-fold greater rate than vehicle. Downmodulation of basal level of genes of the JAK/STAT pathway was confirmed in these cells as well as the inhibition of Stat5 phosphorylation induced by TSLP stimulation. *Conclusions.* Altogheter, our data indicate that Givinostat inhibits the proliferation and induces apoptosis of ALL CRLF2 positive cells bearing or not JAK2 mutations through a specific downmodulation of the genes involved in JAK/STAT pathway and inhibition of their downstream effector molecules. Studies on xenograft models of BCP-ALL leukemia in NOD/SCID mouse are in progress.

C038

DISSECTING THE MOLECULAR MECHANISMS OF ANEUPLOIDY IN ACUTE MYELOID LEUKEMIA

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Introduction. Acute Myeloid Leukemia (AML) is a heterogeneous malignancy characterized by the expansion of myeloid precursor cells with limited or abnormal differentiation capacity. A relatively common event in AML is represented by chromosome gain or loss. Numerical chromosome abnormalities, which define the aneuploid condition, have a detrimental effect in primary non-malignant cells, since they dramatically reduce cellular fitness. However evidence suggests that they have a causative role in tumorigenesis and that they are well tolerated in transformed cells belonging to the myeloid lineage. Aim of the study is to elucidate the pathogenic mechanisms that sustain and contribute to aneuploidy in AML in order to find novel potential therapeutic targets. *Methods.* We have performed gene expression profile analysis of bone marrow cells from 49 AML patients at diagnosis, including 22 aneuploid cases and 27 cases with normal karyotype. All samples contained more than 80% blast cells. The aneuploidy cohort included AML cases carrying one (or more) monosomy, trisomy or a monosomal or complex karyotype. Our analysis covered more than 245,000 and 40,000 coding and non-coding transcripts, respectively (the latter comprising microRNAs), and a significant number of exon-exon junctions, which allow the analysis of multiple splicing isoforms. Quality controls confirmed that the data show comparable signal values. *Results.* The gene expression profile of aneuploid cases has been compared with the one obtained from normal karyotype samples. We have identified a set of coding and non-coding transcripts which are differentially expressed between the two AML subgroups ($p \leq 0.05$, $CV < 0.75$) and belong to functional or operational categories including cell cycle, cell growth and survival, DNA and RNA metabolism, transcription factor and regulator, signaling, cytoskeleton, immune response, migration and adhesion, microRNA. A significant number of the differentially expressed genes could be grouped into Protein Networks, with the largest component comprising 90 genes. Moreover we have identified differences between the two

AML subgroups and normal bone marrow samples. We plan to validate our data by comparing them with published gene expression profile datasets and to perform gene set enrichment analysis. *Conclusions.* By gene expression profile analysis we have identified genes which are commonly and specifically deregulated in aneuploid AML cases and Protein-Protein Interaction Network analysis revealed that many of them are highly connected. This study provides novel insights into the molecular mechanisms sustaining aneuploidy in AML. Future studies on the biological function of selected candidate genes will guide the design of novel therapeutic strategies targeting key players in the disease. *Acknowledgements.* ELN, AIL, AIRC, PRIN, progetto Regione-Università 2010-12 (L. Bolondi), FP7 NGS-PTL project.

C039

FANCONI ANEMIA GENE VARIANTS IN THERAPY-RELATED MYELOID NEOPLASMS

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Introduction. Therapy-related myeloid neoplasms (t-MN) include myelodysplastic syndromes (MDS) and acute myeloid leukemias (AML) occurring as a late effect of chemotherapy and/or radiotherapy for a primary malignancy or for autoimmune diseases. Less than 5% of patients exposed to cytotoxic drugs and radiotherapy develop a t-MN, suggesting an underlying individual susceptibility in this complication. Primary malignancies most frequently associated to t-MN are breast cancer and lymphoproliferative diseases. Fanconi Anemia (FA) syndrome is characterized by chromosomal instability, developmental abnormalities, aplastic anemia and by predisposition to cancer. FA is associated to the occurrence of mutations in the family of FANC genes. *Aims.* Mutations in FANC genes have been rarely described in hematological malignancies outside the syndromic picture of Fanconi Anemia. We were interested in the prevalence of FA variants in t-MN following cytotoxic treatment for breast cancer and lymphoproliferative diseases. *Methods.* The patient cohort included 37 t-MN patients (19 t-MDS and 18 t-AML). The primary malignancy was Hodgkin lymphoma in 7 patients, non-Hodgkin lymphoma in 12 patients and breast cancer in 18 patients. DNA was extracted from BM-MNCs at the time of initial t-MN diagnosis and from different tissues (lymph node biopsies, breast specimens, buccal brush or hair follicles) of the same individuals for the germline controls. Agilent HaloPlex system was used to evaluate the frequency of the SNVs in the selected 14 Fanconi pathway genes [FANCD1 (BRCA2), FANCJ (BRIP1, BACH1), FANC1, FANCA, FANCB, FANCC, FANCD2, FANCE, FANCF, FANCG (XRCC9), FANCL (PHF9), FANCM, FANCN (PALB2) and RAD51C]. All SNVs detected were confirmed using pyrosequencing analysis. The Polyphen-2 tool was consulted to define the putative effect of FA discovered variants. *Results.* FA gene variants were frequent in our t-MN patients, with 6 of 37 patients (16%) carriers of at least one genomic variant, with similar prevalence in t-MN secondary to lymphoproliferative diseases *versus* solid tumors (4 of 19 *vs* 2 of 18, respectively). We found 7 heterozygous FANC variants, including two FANCA (L6F, and S90T), three FANCD2 (T1376A, P256S and M1023V), one FANCJ (I364V) and one FANCC (L36F). Six variants were novel, whereas the FANCA L6F had been previously described. The FANC mutations were germline in all cases (5 confirmed in 37 t-MN patients, 13.5%). Comparing the frequency of FA variants in our t-MN cohort to that reported in *de novo* AML by the Cancer Genome Atlas Research Network, we found a significantly higher frequency of FA SNVs in t-MN patients (13.5% *vs* 2%, Fisher's exact test p=0.005). According to the Polyphen-2 database three of seven FA variants were defined probably or possibly damaging DNA repair. *Conclusions.* Here we report for the first time a high incidence of FA variants in t-MN patients, which may play a role in t-MN susceptibility.

C040

ULTRA-DEEP SEQUENCING STRATEGY IS A PRECIOUS TOOL TO FIND SMALL CLONES HARBOURING FLT3 MUTATIONS IN AML PATIENTS

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Introduction. FLT3 internal tandem duplication (ITD) is frequently detected in AML patients and is an independent predictor of unfavourable outcome, while secondary point mutations in the FLT3 tyrosine kinase domain (KD) are common causes of acquired clinical resistance to FLT3 inhibitors, such as AC220 and Sorafenib. Technologies allowing massively parallel, Ultra-Deep Sequencing (UDS) are currently being evaluated in diagnostic settings since they may conjugate sensitivity and accurate quantification of mutated clones. 886 AML patients were analyzed in Seràgnoli Institute of Bologna between 2002 and 2013 for a panel of genetic alterations including FLT3 ITD and TKD and 239 of 886 (27%) were FLT3+. In particular 256 were cytogenetically normal (CN) AML and of these 66 (25,8%) were FLT3+ and 192 were FLT3-. *Aims.* Since recent whole genome sequencing studies have suggested that FLT3 ITD may evolve from small sub-clones undetectable at diagnosis by routine PCR, we tested the ability of an UDS strategy for FLT3 mutation screening to highlight small clones harbouring ITD mutations. Furthermore, we evaluated if an UDS strategy could highlight in AML patients treated with FLT3 inhibitors emerging clones harboring critical mutations, anticipating the development of drug-resistance. *Methods.* We retrospectively analyzed 2 CN-AML who were found negative for FLT3 ITD- at diagnosis by conventional PCR and Sanger Sequencing, but were then found FLT3 ITD+ during follow-up at relapse or disease progression and 10 AML (5 FLT3+ and 5 FLT3-) treated with the FLT3 inhibitor AC220. In order to reconstruct the dynamic of mutation emergence, we performed a longitudinal re-analysis of RNA samples with UDS on a Roche GS Junior, achieving a lower detection limit between 0,1% and 1%. *Results.* UDS revealed that both the CN-AML analyzed already carried at diagnosis a small clone FLT3 ITD+ (allelic ratio 1-2%), that increased over time during follow-up: in all patients the allelic load of the mutated clone followed then the dynamic of the disease. For the five AML FLT3- treated with AC220 we didn't find any novel FLT3 mutation after treatment, while for the five AML FLT3-ITD+ analyzed, we were able to follow the allelic load of the FLT3-ITD+ clone and the appearance in two patients of novel TKD mutations after treatment that are able to confer drug resistance (allelic ratio 2-55%). *Conclusions.* The high sensitivity of UDS technology is a precious tool to find small clones FLT3 ITD+ that may evolve over time and worsen the prognosis of otherwise good prognosis CN-AML and to highlight emerging mutated clones during treatment with FLT3 inhibitors. For monitoring patients treated with FLT3 inhibitors with UDS we will go on by screening AML treated with Sorafenib, to follow the emergence of any novel critical mutation during treatment. *Acknowledgment.* Work supported by European LeukemiaNet, AIRC, AIL, Programma di ricerca Regione - Università 2010-2012, FP7 NGS-PTL project.

Chronic Lymphoproliferative Disorders II

C041

MODULATION OF ENDOTHELIN 1 EXPRESSION AND INTERACTION WITH B CELL RECEPTOR SIGNALING IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction. Increased evidences suggest that endothelin1 (ET-1) pathway is relevant for tumor growth and dissemination. We have recently demonstrated that the axis of ET-1 and its receptor A (ETAR) is upregulated and induces prolonged survival in chronic lymphocytic leukemia (CLL) cells. Here, we studied the role of gene methylation and some important extracellular stimuli in regulating ET-1 expression in CLL. Moreover, we analyzed the reciprocal interactions between ET-1/ETAR and B cell receptor (BCR) signaling pathways in CLL cells. *Methods.* B cells were purified from peripheral blood of 23 untreated CLL patients and 4 healthy donors (HD). ET-1 methylation status was tested by methylation specific PCR on the first gene intron. ET-1 mRNA expression was valued by Real-Time PCR. CLL cells were cultured in complete medium alone or stimulated with: CpG oligonucleotides+IL-2 (CPG), anti-IgM (IGM), CD40L+IL-4 (CD40). Leukemic cells were treated with idelalisib (PI3Kdelta inhibitor) or PD98059 (MEK inhibitor) with or without ET-1 peptide and/or ETAR specific antagonist BQ-123. BQ-123 was also used on CLL cells previously stimulated by IGM. Apoptosis was studied by flow cytometry using AnnexinV/PI staining and ERK phosphorylation by Western blot. *Results.* ET-1 gene resulted to be methylated (MET) in all HD whereas was unmethylated (UNMET) in 78.3% of CLL patients (p=0.002). In accordance, HD showed lower ET-1 expression than CLL cases (2.06 *vs* 9.35, p=0.005). Among CLL patients, UNMET ET-1 subset had higher ET-1 expression than MET ET-1 subgroup (10.06 *vs* 6.78) and was associated with unmutated IGHV status (p=0.034). After 4 hour stimulation of CLL cells (n=6) with CPG or CD40, we observed an increase of ET-1 expression in all tested patients if compared to untreated samples, with 1.85 and 1.68 mean fold-change, respectively (CPG p=0.043 and CD40 p=0.028). Half of patients responded to IGM with a mean 1.63 fold-upregulation of ET-1 expression. Idelalisib decreased CLL viability from 70±4% to 57±5% (p=0.002). The stimulation with ET-1 peptide reduced idelalisib apoptotic effect (62±4%, p=0.004) that was completely restored by the ETAR blockade (55±5%, p=0.02). Likewise, ET-1 addition increased the viability of CLL cells, previously treated with PD98059, from 64±2% to 69±2% (p=0.001) whereas BQ-123 resensitized leukemic cells to MEK inhibition (viability 64±3%, p=0.006). BCR triggering by IGM induced an increase of CLL viability from 73%±5% to 81%±5% (p<0.001). Interestingly, ETAR blockade by BQ-123 reduced the prosurvival effect of BCR stimulation to 77±6% (p=0.02) and also decreased ERK phosphorylation. *Conclusions.* These results demostrate that: i) higher ET-1 mRNA levels are associated with increased gene demethylation in CLL, ii) ET-1 expression can be rapidly induced in CLL cells by the activation of some important survival and proliferative pathways, iii) ET-1/ETAR and BCR signaling pathways seem to cooperate in inducing anti-apoptotic effects on CLL cells.

C042

MICROENVIRONMENT MODULATION, MICRORNA REGULATION AND PREDICTION POWER OF THE IL23 RECEPTOR (IL23R) COMPLEX IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction. The biology of the CLL cell is influenced by its surrounding immunological and stromal environment. IL23R is a heterodimeric receptor consisting of IL12Rβ1p40 and IL23Rp19 subunits. We investigated 1) IL23Rp19 expression and its correlation with progression free survival (PFS); 2) the role of the microenvironment in controlling the IL23R subunits; 3) the epigenetic regulation of IL23R expression and turnover; 4) the down-stream signaling induced by IL23R engagement in CLL clones. *Methods.* CLL patients were prospectively enrolled at diagnosis (clinicaltrial.gov identifier NCT00917540). MiRNA analysis was performed by Agilent's Human V2 platform and quantitative RT-PCR. The expression of both subunits forming the IL23R complex was determined by flow-cytometry and IHC. MirVANA miRNA mimics and inhibitors, and miRNA target reporter vectors were purchased from Ambion , Inc and Origene, respectively. *Results.* A total of 233 CLL cases were defined according to a median IL23Rp19 cut-off value. The 3-year PFS probability of IL23Rp19-low patients was 91% as compared to 75% of IL23Rp19-high cases [œá2 9.1, P=.003; HR=3.2, 95%CI (1.4-7.1)]. The IL23Rp19-high group was enriched in IGVH unmutated cases with preferential usage of VH1-69 and VH4-34 sequences. Upon multivariate analyses, IL23Rp19 expression still remained significant in predicting PFS together with CD38+ and IGHV mutational status, while ZAP70 and FISH did not. While minimally expressed in circulating B-cells, *in situ* analysis of IL12Rβ1p40 in CLL tissue infiltrates showed a co-expression with IL23Rp19, suggesting that regulation of IL12Rβ1p40 in CLL cells occurred due to microenvironment-derived signals, such as the CD40/CD40L axis. Accordingly, a significant increase in expression of both IL23R subunits occurred co-culturing CLL B-cells in the presence of CD40L. Similar results were obtained stimulating CLL cells with autologous T cells activated by CD3/CD28 MACSiBead™ (Miltenyi) particles and IL2. CLL cells concomitantly expressed higher levels of CD38, lower levels of CXCR4 and CD5dim or bright, thus indicating that the IL23R complex is expressed by activated and dividing B-cells. Both chains of the IL23R complex were expressed in the spleen of a xenografted mouse (NSG) in the context of proliferating Ki67 positive CLL cells. Global miRNA profiling identified miRNAs inversely correlating with IL12Rβ1p40 or IL23Rp19 expression. Dual luciferase reporter assays showed miR-324-5p and miR-146b-5p target the 3'UTR region of IL23Rp19 and IL12Rβ1p40, respectively, which was confirmed in the regulation of the respective proteins in CLL cells overexpressing miR-324-3p or inhibiting miR-146b-5p expression. Finally, the engagement of IL23Rp19 complex induced phosphorylation of Stat3. *Conclusions.* These findings underscore a novel paradigm in the epigenetic regulation of pro-inflammatory cytokine sensing by CLL clones with biological and prognostic relevance.

C043

SURFACE IGM (S-IGM) OR S-IGD PREDICTS DISEASE PROGRESSION AND ARE ASSOCIATED WITH SPECIFIC MIRNA/MRNA SIGNATURES IN CHRONIC LYMPHOCYTIC LEUKEMIA (CLL)

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Introduction. BCR activation stimulates proliferation and survival pathways in CLL cells and is also linked to poor outcome. We investigated sIgM and sIgD 1) expression by flow cytometry, 2) ability to predict progression free survival (PFS) and, 3) miRNA and GEP-associated signa-

tures. *Methods.* 372 patients were prospectively enrolled at diagnosis (clinicaltrial.gov ID NCT00917540). Intensity of sIgM and sIgD expression was calculated as RFI by flow cytometry. Highly purified B-cells were used for GEP (Affymetrix Inc.) and miRNA (Agilent) analyses. *Results.* The average sIgM density was 56.9+5.6 RFI (mean+sem) across the entire cohort, with a median RFI=34 (range 6.7-1430). ROC analysis determined sIgM RFI=10 as the best threshold to distinguish IgMlow cases (234/372, 62.9%) from sIgMhigh (AUC=0.629, P<.0001). The sIgMlow group had a significantly longer PFS than sIgMhigh cases (H.R. 2.2, 95%CI 1.4-3.5, P<.0001). Similarly, using a RFI=12 threshold value for sIgD, the 86 cases with sIgDhigh had a significantly higher progression risk (HR=2.4, 95%CI 1.5-3.8, P<.0001). Among the 234 sIgMlow cases there was a concordantly low sIgD density in 194 cases, while 46/138 of sIgMhigh cases exhibited higher sIgD RFI levels (P=.001).Subdividing cases into 3 groups [sIgMlowsIgDlow (n=194), sIgM or sIgDhigh (n=132), and sIgMhigh/sIgDhigh (n=46)], significant differences in clinical outcome were observed between sIgMlowsIgDlow, sIgM or sIgDhigh, and sIgMhigh/sIgDhigh cases with 4.5 times higher progression risk (95%C.I. 2.5-8.1, P<.0001) for the latter group. In a Cox multivariate model, CD38+ [HR=2.2, 95%CI 1.2-3.8, P=.006], IGHV-UM [HR=3.0, 95%CI 1.6-5.6, P=.001] and sIgMhigh/sIgDhigh cases [HR=2.1, 95%CI 1.2-4.0, P=.016] maintained an independent association with PFS. A GEP multiclass supervised analysis for the three sIgD and sIgM density categories identified 23 differently modulated genes; 18/23 could predict PFS. Finally, a miRNA multiclass supervised analysis identified 4 differently modulated miRNAs. Specifically, miR-146b-5p was up-regulated from sIgDlow/sIgMlow to sIgDhigh/sIgMhigh groups, while the opposite was observed for miR-575, miR-1225-5p and miR-373*. Only miR-146b-5p (P=.011) was significantly associated with PFS. *In vitro* stimulation of CLL cells by autologous T cells activated and expanded by CD3/CD28 MACSiBead™ (Miltenyi) particles and IL2, up-regulated both sIgD and sIgM expression in a subset of B-cells also showing CD38bight/CXCR4dim/CD5bright. *Conclusions.* Higher cell surface density of IgM and IgD correlates with shorter PFS independently of other known prognostic markers. Moreover, genes and miRNAs are differently regulated in cases with high sIgD and sIgM expression and predict a poor clinical outcome, thus stressing the relevance of sIgD and sIgM regulation in the natural history of early unfavorable CLL patients. Finally, microenvironmental signals have a role in BCR regulation.

C044

MICRORNAOME EXPRESSION IN CHRONIC LYMPHOCYTIC LEUKEMIA: COMPARISON WITH NORMAL B CELL SUBSETS AND CORRELATIONS WITH PROGNOSTIC AND CLINICAL PARAMETERS IN 217 PATIENTS INCLUDED IN THE OBSERVATIONAL PROTOCOL O-CLL1

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Introduction. Despite its indolent nature, chronic lymphocytic leukemia (CLL) remains an incurable disease. A pathogenetic role of miR-15/16 was evidenced in CLL and the involvement of additional deregulated microRNAs (miRNA) was suggested. However, the pattern of deregulated miRNAs in CLL could be related to a number of factors, including presence/absence of chromosomal abnormalities, disease stage and importantly, to the types of compared normal B-cells. *Methods.* We analyzed the expression of mature miRNAs respectively in 217 early stage CLL cases included in the observational O-CLL1 protocol and in various different normal B-cell subpopulations, from tonsils and peripheral blood. High quality total RNA samples were hybridized on Agilent Human miRNA microarray, investigating 723 human miRNAs (Sanger miRBase, release 10.1). MicroRNA expression profiles were obtained according to the manufacturer's procedure; quantile normalization was applied. Microarray results were analyzed using the GeneSpring GX software (Agilent Technologies) or the Qlucore Omics Explorer (Qlucore, Lund, Sweden). *Results.* Unsupervised analyses indicated that CLL cells exhibited a miRNA expression pattern that was most similar to the subsets of antigen-experienced and marginal zone-like B-cells. Normal subpopulations were used as a reference to identify differentially expressed miRNAs in comparison with CLL. Differences related to the expression of 25 miRNAs were found to be independent from IGHV mutational status or cytogenetic aberrations. These differences, confirmed in an independent validation set, led to a novel comprehensive description of miRNAs potentially involved in CLL. We also identified miRNAs whose expression was distinctive of cases with mutated *versus* unmutated IGHV genes or cases with 13q, 11q, and 17p deletions and trisomy 12. Finally, analysis of clinical data in relation to miRNA expression revealed that miR-26a, miR-532-3p, miR-146-5p and miR-29c* were strongly associated with progression free survival. *Conclusions.* This study provides novel information on miRNAs expressed by CLL and normal B-cell subtypes, with implication on the cell of origin of CLL. In addition, our findings indicate a number of deregulated miRNAs in CLL, which may play a pathogenic role and promote disease progression. Collectively, this information can be utilized for developing miRNA-based therapeutic strategies in CLL.

C045

CK2 EXPRESSION AND INHIBITION IN GERMINAL CENTER-RELATED LYMPHOMAS

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Introduction. protein kinase CK2 deregulation has been consistently documented in several solid and hematological tumors. CK2 overexpression has been reported in T- and B-cell acute lymphoblastic leukemia, B-cell chronic lymphocytic leukemia, mantle cell lymphoma and multiple myeloma. Of note, CK2 expression has been recently demonstrated also in non-neoplastic germinal centers (CG) of reactive lymph nodes, but its expression in GC-related lymphoproliferative disorders has never been investigated. *Methods.* Immunohistochemical expression of both CK2α and CK2β subunits was performed in a series of 101 GC-related non-Hodgkin lymphomas: 53 follicular lymphomas (FL); Bcl2-positive FL=28 cases; Bcl2-negative FL=25 cases, 18 sporadic Burkitt lymphomas (BL) and 30 diffuse large B-cell lymphomas (DLBCL) of B-cell germinal center type (BGC-DLBCL) were considered. To evaluate CK2 expression in the whole spectrum of DLBCL subtypes, an additional series of 22 DLBCLs of non-activated B-cell-type (ABC-DLBCL) was also included. Western blot also evaluated CK2 in BL and DLBCL cell lines. The clinical-grade, orally bioavailable CK2 inhibitor CX-4945 was tested on NHL cells. *Results.* Immunohistochemistry revealed that both CK2 subunits were diffusely expressed in all the considered cases. In particular, a moderate to strong positivity (score ≥2+) was documented in more than 87% of CG-derived lymphomas and DLBCLs (CK2α score ≥2+: 87.8% [108/123]; CK2β score ≥2+: 88.6% [109/123]). No cases showed negative staining for either antigen. However, compared to FLs and DLBCLs, BLs were more frequently associated with moderate-to-strong CK2α expression (trend of association, Fischer exact test). In particular, a CK2α score≥2+ was reported in 97.1% [17/18] of BLs and in 84.9% (45/53) and 88.4% (49/52) of FLs and DLBCLs, respectively. CK2 subunits were equally expressed in Bcl2-positive and Bcl2-negative FLs. In particular, moderate-to-strong positivity for CK2α was reported in 85.7% (24/28) of Bcl2-positive and in 84% (21/25) of Bcl2-negative FLs (no statistically significant differences, Fisher exact test). Similar results were obtained for the CK2β subunit. As for DLBCLs, CK2 subunits were more strongly expressed in ABC- than

in BGC-cases. In particular, a CK2α score ≥2+ was documented in 90.9% (20/22) of ABC-DLBCLs and in 86.7% (26/30) of BGC-DLBCLs. Likewise, CK2β subunit was moderately-to-strongly expressed in 95.5% (21/22) of ABC-DLBCLs and in 90.0%(27/30) of BGC-DLBCLs (Figure 1). CK2 expression did not correlate with Myc/Bcl2 status. Moreover, BL, ABC-DLBCL and BGC DLBCL cells displayed a significant sensitivity to the cytotoxic effect of CX-4945. *Conclusions.* These data show that CK2 is overexpressed in GC-derived and ABC-type NHLs suggesting a biological and pathogenic role in the GC. Moreover, the availability of CK2 inhibitors already in the clinical scenario allow envisioning a place for this kinase in the therapeutic targets for NHLs.

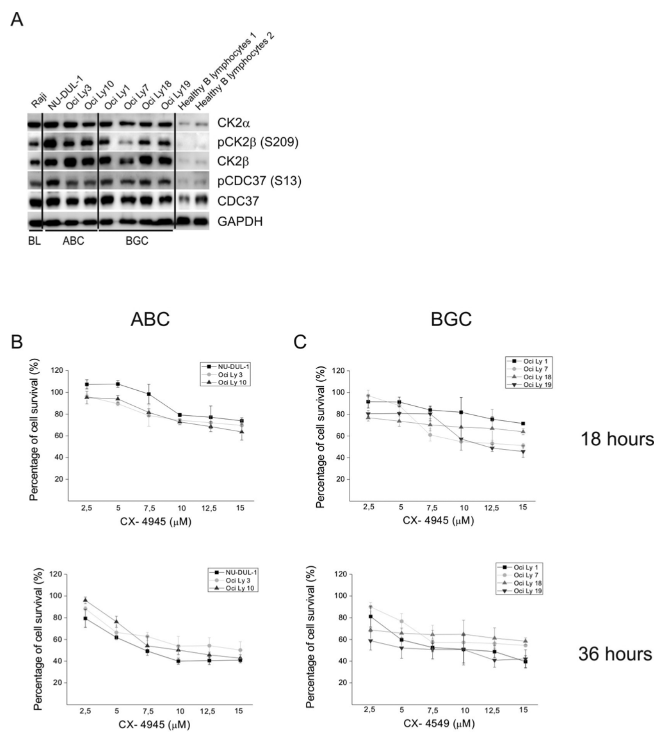

Figure 1.

C046

IBRUTINIB NAÏVE CHRONIC LYMPHOCYTIC LEUKEMIA LACKS BRUTON'S TYROSINE KINASE MUTATIONS ASSOCIATED WITH TREATMENT RESISTANCE

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Introduction. The Bruton's tyrosine kinase (BTK) inhibitor ibrutinib blocks B-cell receptor (BCR) signaling via covalent binding of the C481 residue of BTK. Though ibrutinib induces durable remissions in relapsed/refractory chronic lymphocytic leukemia (CLL), a small fraction of patients treated with this targeted therapy still develop progressive disease after an initial response. Genomic studies disclosed mutations affecting the C481 codon of BTK in a sizeable fraction of ibrutinib-resistant CLL. These mutations interfere with the function of ibrutinib by blocking its covalent binding to BTK, and have been observed in patients harboring prior poor risk cytogenetic lesions (*i.e.* 17p deletion). In other models of tyrosine kinase inhibition (TKI), resistant mutations can be identified since the early phase of the disease in a small fraction of the tumor clone before exposure to the selective pressure of TKI. *Methods.* Here we assessed the occurrence of small subclones harboring the C481S codon mutations (*i.e.* c.T1441A; c.G1442C) of BTK in ibrutinib na√Øve CLL patients. Mutation analysis was performed by allele-specific PCR (AS-PCR) tailored at a sensitivity of 10-3 (i.e detection of one mutant allele/1000 wild type alleles). *Results.* The study cohort comprises 553 newly presented CLL (151 with TP53 abnormalities), 30 progressive and fludarabine refractory CLL (12 with TP53 abnormalities) and 30 Richter syndrome (15 with TP53 abnormalities). All patients were ibrutinib na√Øve. By AS-PCR, neither newly presented CLL, nor progressive fludarabine refractory CLL or Richter syndrome harbored BTK C481S mutated clones above the sensitivity threshold of the assay. In order to validate this observation with an independent platform, 24 ibrutinib na√Øve CLL harboring TP53 disruption, who seem to be at higher risk of developing BTK variants, were also investigated by ultra deep next generation sequencing of the BTK mutation hotspot using the 454 chemistry. The target coverage was ~10000x per amplicon to obtain a sensitivity of 10-3 (i.e detection of one mutant allele/1000 wild type alleles). This approach confirmed that none of the 24 TP53 disrupted CLL harbored BTK C481S mutated clones. *Conclusions.* Overall, these data indicate that, among CLL that have not been exposed to ibrutinib, the BTK C481S variant conferring resistance to this drug is absent or limited to a subtle fraction of the clone that cannot be resolved with the current approaches. In this respect, CLL differs from chronic myeloid leukemia (CML), another model of mutation-driven resistance to TKI. At variance of ABL mutations of CML, ibrutinib-resistant BTK mutations in CLL: i) are selected to affect one single codon to witch ibrutinib covalent binds; and ii) are irrelevant to the CLL clone in the absence of selective pressures imposed by ibrutinib.

C047

COOPERATION BETWEEN ADENOSINERGIC AND HYPOXIC SIGNALS IN SHAPING CHRONIC LYMPHOCYTIC LEUKEMIA MICROENVIRONMENT

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Extracellular adenosine generated from ATP/ADP through the concerted action of the ectoenzymes CD39 and CD73 elicits potent cytoprotective and immunosuppressive effects mediated by type-1 purinergic receptors. Chronic lymphocytic leukemia (CLL) patients expressing the ectoenzymes CD39 and CD73 can actively produce adenosine. This condition activates an autocrine adenosinergic axis that supports engraftment of leukemic cells in a growth-favorable environment. These effects are mediated by the A2A adenosine receptor, which inhibits chemotaxis and limits spontaneous and drug-induced apoptosis of CLL cells. Following the reported cross-talk between hypoxia and adenosine, we tested the hypothesis of a functional interplay between the adenosinergic axis and hypoxic signals in the CLL microenvironment. Results confirm that the CLL cells robustly increase HIF-1α expression when cultured under low oxygen tension. Under these conditions a significant increase in the mRNA and protein levels of CD73, CD26 and of A2A was observed. An HPLC assay confirmed that hypoxic CLL cell cultures are characterized by higher extracellular adenosine levels, further improved upon inhibition of adenosine deaminase and nucleoside transporters. Attention was then concentrated on the stromal compartment, which is critical to the formation and maintenance of the leukemic niche. Here, hypoxia enhanced differentiation of circulating monocytes into nurse-like cells, macrophages of the M2 type, which play an essential role in nurturing leukemic cells. During hypoxic culture, differentiating monocytes up-regulated A2A and A3. Both receptors were overexpressed by NLC under hypoxic conditions. Furthermore, they were functional, as determined by the finding of increased AKT and ERK1/2 phosphorylation following pharmacological activation of the receptors. The enhancement of NLC differentiation under hypoxic conditions relied, at least in part, on the activation of A2A and A3: their engagement using agonists enhanced NLC differentiation in normoxia, with overexpression of IDO, CD163 and CD206. Furthermore, activation of A2A and A3 favored secretion of immunomodulatory cytokines such as IL-10 and IL-6. On the contrary, their pharmacological blockade under hypoxia prevented NLC differentiation. Together, these results indicate that the adenosinergic and hypoxic axes synergize in shaping the CLL niche, suggesting that the pharmacological inhibition

of adenosinergic signals may counteract some of the effects mediated by an hypoxic microenvironment.

C048

THE CODING GENOME OF NODAL MARGINAL ZONE LYMPHOMA REVEALS RECURRENT MUTATIONS OF NOTCH, NF-κB AND CHROMATIN REMODELING GENES

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Introduction. Among marginal zone lymphoma (MZL), splenic MZL (SMZL) and nodal MZL (NMZL) share similar morphology and phenotype and are distinguished solely by different patterns of dissemination. Unbiased genomic studies have unraveled the typical coding genome of SMZL, which is characterized by mutations of genes involved in the physiological differentiation of MZ B-cells, including mutations of NOTCH2 and other NOTCH pathway genes in 40% of cases, NF-κB pathway mutations in ~25%, and disruption of the chromatin remodeler gene MLL2 in 15% of cases. Conversely, NMZL still remains orphan of specific genetic lesions. The project aims at characterizing the coding genome of NMZL to improve future classification of MZL and provide novel biomarkers for the refinement of their diagnosis. METHODS. Paired tumor/normal DNA from 18 discovery NMZL were analyzed by whole exon sequencing (WES) and SNP array (Cytoscan, Affymetrix). Gene mutation recurrence was validated in an independent panel of NMZL (n=22). In all cases, NMZL diagnosis was confirmed by centralized pathological revision of lymph node histology (IRTA1 expression in 60% of cases), and by the lack of clinico-radiological evidence of extranodal or splenic disease either at diagnosis or during follow-up. *Results.* WES and SNP array identified 557 non-synonimous somatic mutations (average: 30.8/case) and 51 copy number abnormalities (CNA) (average 3.2/case), respectively, in the 18 discovery NMZL. Mutations were predominantly missense substitutions (82%) and infrequently frameshift/in frame deletions. Mutations affected 504 genes, including 28 genes recurrently mutated in >1 patient. To verify the mutation frequency of the discovered genes, they were further investigated in an independent validation panel of 22 NMZL. By compiling the results of the discovery and validation panels, the most typical mutations of NMZL were disrupting events of NOTCH2 (27.5%), MLL2 (26.6%), TNFAIP3 (15%) and BCL10 (16.6%). To gain a comprehensive outlook of the CNA profile of NMZL, along with cases of the discovery panel, we examined 17 additional samples from the validation panel (total number=35) by SNP arrays. CNA were preferentially represented by deletions (60.8%), including focal losses encompassing the TRAF3 (22.8%, including 1 case harboring a disrupting mutation in second allele), CDKN2A (11.4% including 1 homozygous deletion), PTEN (8.6%) and TNFAIP3 (5.7%, including 1 case with homozygous deletion) genes. *Conclusions.* Overall, the genetics of NMZL indicates the involvement of the NOTCH, NF-κB and chromatin modification pathogenetic pathways that are also largely implicated in SMZL, suggesting that NMZL and SMZL represent different clinical presentations of the same disease entity. These data bear implications for a genetics-driven classification and diagnosis of MZL as well as for identifying molecular targets for therapy of these lymphomas.

Monoclonal Gammopathies and Multiple Myeloma II

C049

TARGETING ANGIOGENESIS IN MULTIPLE MYELOMA: A POSSIBLE ROLE FOR EphA3 AND A SPECIFIC MONOCLONAL ANTIBODY

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Introduction. Multiple myeloma (MM) remains an incurable malignancy despite important recent advances in treatments. Neo-vascularization entails a crucial aspect of interactions between neoplastic plasma cells (PCs) and their microenvironment (ME). To overcome drug resistance and improve clinical response to novel therapeutic approaches halting both PC growth and the increased bone marrow (BM) microvascular density are needed. In this setting, monoclonal antibodies against MM-specific cell surface antigens represent a promising therapeutic approach, which is however hampered by a lack of appropriate membrane target structures expressed across all MM cells. The Eph receptors, a large family of receptor tyrosine kinases, have been implicated in many processes involved in malignancy, including alteration of the tumour ME, and in angiogenesis, in both of which EphA3 likely plays an active role. A engineered antibody targeting the EphA3 was developed and it is now under phase I clinical trials in USA and Australia for the treatment of EphA3 over-expressing hematological myeloid malignancies refractory to conventional treatment. We investigate the role of EphA3 in MM angiogenesis and the effect of its specific monoclonal antibody to define EphA3 as possible therapeutic target in MM.

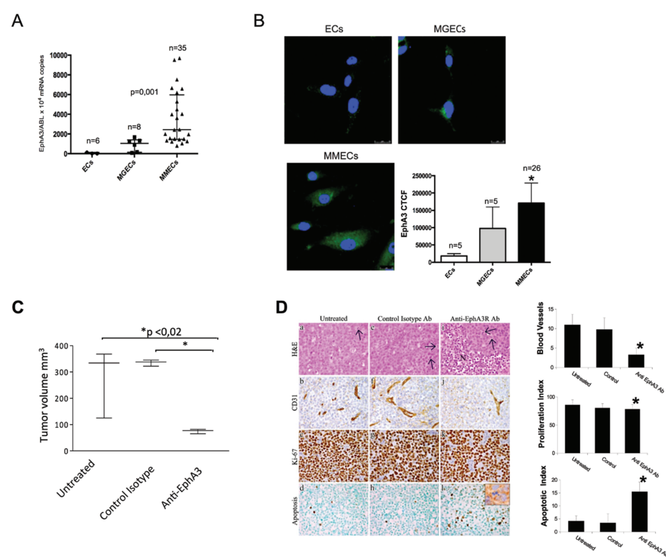

Figure 1.

Methods. EphA3 mRNA and protein were evaluated in ECs of MM patients (MMECs), patients with monoclonal gammapathies of undetermined significance (MGECs) and patients with benign anemia (ECs controls) by absolut RT-PCR and by western blot coupled to immunofluorescence and FACS analysis, respectively. Immunoistochemistry was also performed on MM BM biopsies. The expression of EphA3 proangiogenic targets in silincing MMECs was studied by gene expressionn profiling. The effects of EphA3 targeting by siRNA or by the anti EphA3 specific antibody on the angiogenesis was evaluated *in vitro* and *in vivo*. The antitumor activity of the antibody targeting EphA3 was measured *in vivo* in MM xenograft mice. *Results.* Briefly, our data showed that EphA3 mRNA and protein levels are progressively increased from ECs to MGECs, reaching the highest values in MMECs. (Figure 1 A-B) EphA3 stained intensely and diffusely MM microvessels and PC in MM BM biopsies. The EphA3 targeting by either siRNA or anti EphA3 Ab impaired the MMECs angiogenesis related functions both *in vitro* and *in vivo*. In particular, tumour masses developed in xenograft mice treated with anti-EphA3 Abs were smaller in size (Figure 1 C) and showed foci of ischemic-hemorrhagic necrosis, in association with a significant ($p < 0.05$) reduction in the number of intact tumor microvessels compared to the mice treated with control isotope antibody (Figure 1 D). *Conclusions.* EphA3 plays a critical role in MM angiogenesis; the anti EphA3 antibody inhibits this process *in vitro* and *in vivo*. EphA3 targeting could represent a possible strategy for the treatment of MM patients.

C050

HIGH-THROUGHPUT SEQUENCING FOR THE IDENTIFICATION OF DIS3 MUTATIONS IN MULTIPLE MYELOMA

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Introduction. DIS3 is a catalytic subunit of the human exosome complex, containing exonucleolytic (RNB) and endonucleolytic (PIN) domains. Recently, whole exome sequencing studies have identified DIS3 mutations in about 10% of patients with multiple myeloma (MM). *Methods.* To analyze DIS3 mutation in untreated MM, we investigated by next generation sequencing (NGS) a retrospective cohort of 96 cases at onset, seven of whom were also tested at relapse. Moreover, we examined 11 patients with secondary plasma cell (PC) leukemia (sPCL). Deep sequencing of the PIN and RNB domains was performed by Roche 454 pyrosequencing on the Genome Sequencer Junior instrument. Mutations were validated by conventional Sanger sequencing. *Results.* NGS analysis revealed the presence of 49 coding non-synonymous variants, all of which but one were confirmed as somatic by sequencing the normal matched DNA. Mutant allele frequency ranged from 0.26% to 100% of total reads (median depth of coverage 264x, range: 102-870). Among the 48 tumor-specific mutations, 33 (69%) were single nucleotide variations, and the remaining 15 (31%) were indels. At the amino acid level, 32 mutations (67%) were missense, three (6%) nonsense, 11 (23%) introduced a frameshift, and two (4%) an in-frame deletion. Seven of these variants have been already reported by others, also specifically in MM patients, while 41 were novel. The great majority of identified mutations affected the RNB domain (41, 85%). The occurrence of the mutations was subsequently assessed by Sanger sequencing, which allowed confirming the presence of variants in the 14 cases with frequency of mutated sequencing reads greater than 24%; specifically, they affected 11 MM patients at diagnosis (11/96, 11.5%) and three sPCL cases (3/11, 27.3%). In the three mutated samples analyzed also at relapse, DIS3 mutation status was confirmed as found at diagnosis; notably, in one mutated case, carrying DIS3 R467Q mutation, the variant allele frequency increased from 31% to 82%. Three mutations (D488N, R780K, and R789W) were recurrent, each affecting two patients; furthermore, a sPCL sample displayed two mutations in the RNB domain (E626K and R780K), and a MM patient harbored a 9aa in-frame deletion in the PIN domain (Ala47_Pro55del) and a missense substitution in the RNB domain (A751D). DIS3 mutations were not associated with the occurrence of IGH translocations nor with del(13). Mutant allele frequency was >90% only in 44% of the patients positive for del(13); in all the remaining cases, the percentage of variant reads was suggestive of a mutation present in heterozygosis or in a small tumor subclone.*Conclusions.* Our data confirm DIS3 as frequently mutated in MM, and importantly, seem to indicate an even greater involvement of DIS3 alteration in more advanced stages of PC dyscrasias. Further studies are required to elucidate the role of this gene in the pathogenesis of MM, as well as its potential use as a drug target.

C051

UPDATED RESULTS OF BENDAMUSTINE, BORTEZOMIB AND DEXAMETHASONE (BVD) IN PATIENTS WITH RELAPSED-REFRACTORY MULTIPLE MYELOMA (MM)

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Introduction. In patients with MM, Bendamustine demonstrated efficacy and tolerability as monotherapy or in combination with new drugs. *Methods.* Here we present the updated results of a prospective, phase II study conducted in 22 Italian centres of the combination Bendamustine (70 mg/m2 days 1, 8), Bortezomib (1.3 mg/ m2 days 1, 4, 8, 11) and Dexamethasone (20 mg days 1-2, 4-5, 8-9, 11-12) (BVD) administered every 4 weeks in patients with relapsed-refractory MM of any age, with adequate cardiac, liver and hematological function, not refractory to bortezomib and treated with no more than four previous lines of therapy. The primary endpoint was achievement of a response at least PR, as to IMWG criteria, after four cycles of BVD. Patients obtaining at least a PR received two additional treatment cycles followed by a 12-months consolidation phase with cycles repeated every 2 months. Patients achieving a response less than a PR were taken off-study. *Results.* 75 patients were included. Median age was 68 years (range 41-85), 26.5% had ISS stage 3, 19% IgA myeloma and 9% renal failure. Eight of 36 evaluable patients (22%) had adverse cytogenetic. Patients had received a median of one prior line of therapy (range 1-4). All patients had received prior treatment with new drugs, such as thalidomide (57%), lenalidomide (54.5%) or bortezomib (46.5%) or both (20%). Twenty-four patients (32%) were refractory to IMIDs. Best response rate was 75%, including 14 CRs (20%), 22 VGPRs (24%) and 27 PRs (31%). Five patients (6.5%) died early. Only prior treatment with bortezomib significantly reduced the response rate ≥ PR (48.5% *vs* 80%; P=0.004). At a median follow-up of 27 months (range 18-38), 45 patients had progressed and 43 had died. Median TTP and PFS were 17 and 12.5 months, respectively while median OS was 24 months (40% at 3 years). After longer follow-up, prior therapy with bortezomib plus lenalidomide was con-

firmed as the only factor that significantly reduced TTP (9 *vs* 19 months; HR=2.7; 95% CI=1.3-5.8; P=0.009), PFS (9 *vs* 15 months; HR=2.1; 95% CI=1.2-3.8; P=0.020) and OS (17 *vs* 32 months; HR=2.1; 95% CI=1.2-3.9; P=0.043). Grade 3-4 adverse events occurred in 55% of patients leading to therapy reduction in 24% and to protocol discontinuation in 11% of patients. The most frequent severe adverse events were thrombocytopenia (28%), neutropenia (20%), infections (12%), peripheral neuropathy (9%), gastrointestinal (5%) and cardiovascular events (4%). Compared with younger, patients aged > 70 years had a significantly higher incidence of grade 3-4 thrombocytopenia, infections and consequently a higher rate of therapy reduction and discontinuation. Moreover, 4/5 early deaths occurred in patients aged more than 70 years. *Conclusions.* BVD combination is an effective and well tolerated regimen in relapsed-refractory MM. Data suggest that the optimal target of BVD maybe patient younger than 70 years prior treated with bortezomib or lenalidomide.

C052

GENE EXPRESSION PROFILING AND COPY NUMBER ALTERATIONS OF CLONOTYPIC B CELLS OF MULTIPLE MYELOMA NEWLY DIAGNOSED PATIENTS REVEALS PATHWAYS POTENTIALLY INVOLVED IN THE DISEASE PERSISTENCE

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Although the advances in Multiple Myeloma (MM) therapy, the disease remains incurable. The persistence of an immature cells population, residing in the CD138- cell compartment and resembling the memory B cells, has been supposed to be one of the major causes of MM drug-resistance. Aim of the study is to evaluate the genomic and genetic background of MM immature B cells, located both in bone marrow (BM) and in peripheral blood (PBL), as compared to the neoplastic plasmacells. CD138+/CD138- cell fractions were collected from 50 newly diagnosed MM patients, and CD138-19+/CD138-19+27+ cell fractions were isolated. Clonogenic assays were performed using cell fractions obtained from RPMI-8226 and NCI-H929 cell lines. For each cell fraction, we performed a sequencing of the IgH rearrangement. The complete set of genomic aberrations and the gene expression profiles were performed by using the SNPs array 6.0 and the HG-U133 Plus 2.0 array (Affymetrix). Clonogenic assays showed that the CD138- cells, plated in conditioned media, were more able to form colonies, as compared to the CD138+ ones. By IgH rearrangement analyses, a clonal relationship between the CD138+ and the memory B cells clones was confirmed. SNPs arrays showed that both BM and PBL CD138+ cell fractions displayed exactly the same genomic macro-alterations. On the contrary, in the BM and PBL CD138-19+27+ memory B cell fractions any macro-alteration was detected, whereas several micro-alterations were highlighted. These micro-alterations were located out of any genomic variant regions and were presumably associated to MM pathogenesis, since KRAS, WWOX and XIAP genes are located among the amplified regions. In addition, several LOH regions were highlighted in memory B cells, which cover at least 106 tumor suppressor genes (among which are TP53, CDKN2C and RASSF1A), already known to be involved in MM and other hematological malignancies. To get insight into the biology of the clonotypic B cell population, the gene expression profiles of 5 donor B cells and 11 MM B cells samples were compared. Unsupervised analysis by hierarchical clustering was able discriminate the differential expression of 11480 probes (FC: <-2;>2; FDR: 0,05; p <0,05). An overall de-regulation of pathways involved in self-renewal mechanisms was observed (down-regulation of Hedgehog pathway; over-expression of Notch and Wnt signaling). In addition, the down-regulation of genes related to the unfolded protein response (such as IRE1α and XBP1: -18.0; -19.96. p<0,05) supports the hypothesis of an intrinsic proteasome inhibitors' resistance. Data suggest that the MM CD138+ clone might resume the end of the complex process of tumorigenesis. In contrast, MM B cells, which lack macro-alterations, display several micro-alterations and a peculiar transcriptional program, thus supporting the idea that these post-germinal center cells might be involved in the transforming event, which originate and sustain the neoplastic clone.

C053

GRANULOCYTIC MYELOID DERIVED SUPPRESSOR CELLS ARE INCREASED IN MULTIPLE MYELOMA AND CAN PREDICT OUTCOME IN PATIENTS TREATED UPFRONT WITH NOVEL AGENTS

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Introduction. Recent studies have indicate a role for the myeloid compartment in the biology of Multiple Myeloma (MM): the neutrophil to lymphocyte ratio (NLR) and the lymphocyte to monocyte ratio (LMR) are emerging predictors of progression free survival (PFS) and overall survival (OS) in MM. In addition, Myeloid-derived suppressor cells (MDSC), a heterogeneous population of myeloid cells with peculiar immunosuppressive properties against T-cells, are increased in MM, as recently described. *Aims.* We investigated MDSC and clinical variables at diagnosis (including NLR and LMR) and their impact on outcome of patients treated upfront with novel agents (lenalidomide and bortezomib). *Methods.* We evaluated by flow cytometry G-MDSC (CD11b+CD33+CD14-HLADR-) in 45 newly diagnosed MM patients treated upfront with lenalidomide (N=30) or bortezomib (N=15), compared to 50 MGUS and 30 healthy subjects matched for sex and age. We tested the immunosuppressive activity of G-MDSC by the expression of the enzyme arginase (ARG-1) and ability to suppress lymphocytes activation when co-cultured with T-lymphocytes obtained from normal donors. *Results.* G-MDSC percentage in MM was greater than healthy controls (61.2±1.6% *versus* 51.7±1.4%, p<0.001) with a large overlapping with granulocytes. We found a T-cell anergy dose- and time-dependent driven by MM-granulocytes. Consistent with this finding, MM- granulocytes overexpressed ARG-1 (p=0.0012), being functionally G-MDSC. G-MDSC were higher in presence of extensive bone disease (p=0.02), in patients carrying LDH ≥2 UPN (p=0.0025) and fibrinogen ≥40 mg/dL (p=0.01). G-MDSC were also positively correlated to neutrophil to lymphocyte ratio (NLR) and lymphocyte to monocyte ratio (LMR), recently described as novel biomarkers in MM (respectively, r2=0.60, p<0.0001, and r2=0.26, p=0.01). G-MDSC were reduced after exposure to lenalidomide-based regimen (p<0.0001) but not to bortezomib. Patients treated upfront with lenalidomide and G-MDSC >60% at diagnosis had shorter progression free survival (PFS) than those with less than 60% (16.8 *versus* 47.0 months, p=0.04). *Conclusions.* G-MDSC are increased in MM; they can predict response to lenalidomide. NLR and LMR are surrogates for G-MDSC and this can explain their prognostic meaning recently reported.

C054

THE ANTI-MYELOMA EFFECT OF LENALIDOMIDE IS INCREASED BY HYPOXIA-INDUCIBLE FACTOR (HIF)-1a INHIBITION IN MULTPLE MYELOMA CELLS BOTH *IN VITRO* AND *IN VIVO*

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Introduction. We have recently shown that HIF-1α is overexpressed by multiple myeloma (MM) cells and HIF-1α suppression significantly blocks MM-induced angiogenesis and reduces the MM tumor burden and bone destruction *in vivo*. The potential effects of HIF-1α on drug sensitivity on MM cells are not known and have been investigated in this study. *Methods.* Firstly, we explored the *in vitro* effect on cell proliferation, survival and pro-angiogenic profile of the treatment with the immunomodulatory drug (IMiD®), Lenalidomide (LEN) (0,2-100 μM), and the proteasome inhibitor Bortezomib (BOR) (2-10 nM) in the human MM cell line JJN3 with or without a stable HIF-1α inhibition obtained by anti-HIF-1α lentiviral shRNA pool. Cell proliferation and viability were checked by 3H-Thymidine uptake and MTT assay,

respectively. The effect of LEN in combination with HIF-1α inhibition was then assessed *in vivo* in a NOD/SCID subcutaneous mice model. Different groups of animals, injected with JJN3-PLKO.1 (empty vector) or JJN3-anti-HIF-1α, were treated with LEN (5mg/kg) using the intraperitoneal route. After three weeks, we evaluated tumor volume and weight and microvascular density, checked by CD34 immunostaining. The expression of the main cell proliferation and survival signaling molecules p27, IGF1R, IRF4, MCL1, Caspase 3, SKP2, Hexokinase, pERK 1/2, GRP78, CHOP were evaluated by Western Blot in both *in vitro* treated JJN3 and in tumor masses *in vivo*. *Results.* Treatment with LEN induced a significant higher inhibition of cell proliferation in JJN3 anti-HIF-1α as compared to JJN3 pLKO.1 (P=0.01) but not of cell viability whereas BOR induced a similar rate of cell proliferation and cell death in both JJN3 anti-HIF-1α and JJN3 pLKO.1. Consistently, we found that LEN increased p27 expression and down-regulated IRF4 expression in JJN3 anti-HIF-1a as compared with JJN3 pLKO.1. A down-regulation of VEGFA mRNA expression was observed in JJN3 anti-HIF-1α as compared to JJN3 pLKO.1 after LEN treatment; on the other hand, other pro-angiogenic molecules were paradoxically up-regulated such as CCL2 and IL8. In the *in vivo* mouse model, we found that LEN treatment induced a dramatic reduction of the weight and volume of the tumor burden in mice injected with JJN3 anti-HIF-1α as compared to JJN3-pLKO.1. The Western Blot analysis on tumor masses showed a down-regulation of Hexokinase and IGF1R by HIF-1α inhibition and the treatment with LEN further suppressed the expression of IRF4. Finally, the microvascular density was inhibited by HIF-1α inhibition but not further reduced by LEN treatment. *Conclusions.* Overall our data indicate that HIF-1α suppression in MM cells significantly increase the anti-MM effect of LEN reducing the MM cell growth *in vitro* and *in vivo*, through the inhibition of proliferation and survival signaling molecules. These data suggest that the combination of LEN and HIF-1α inhibition have a therapeutic rationale in MM.

C055

DIFFERENT GENE AND MIRNA EXPRESSION PROFILES IN PRIMARY AND SECONDARY PLASMA CELL LEUKEMIA BY HIGH-RESOLUTION MICROARRAY ANALYSES

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Introduction. Plasma cell leukemia (PCL) is a very aggressive and rare hematologic malignancy that can be distinguished into primary (pPCL), originating *de novo*, or secondary (sPCL) malignancy, arising as a leukemic transformation of multiple myeloma (MM). Global gene (GEP) and miRNA expression profiles were generated in pPCL and sPCL patients, in order to elucidate the molecular features of these two clinical forms. *Methods.* GEP of highly purified plasma cells (PCs) from 23 pPCLs, enrolled in an Italian GIMEMA multicenter clinical trial, and 11 sPCL patients were generated on Affymetrix Gene 1.0 ST array. Fifteen pPCLs and 10 sPCLs were also analyzed on miRNA 3.0 array. Hierarchical clustering and supervised analyses were carried out by dChip and SAM software, respectively. Functional annotation studies were performed using DAVID 6.7 and miRBase tools. Main genomic aberrations were investigated by FISH analysis. GEP were also available in our laboratory in a series of 121 untreated MMs at onset, 18 human myeloma cell lines (HMCLs) and 4 normal bone-marrow PCs samples. *Results.* Hierarchical clustering of the most variable genes on PCL cases clearly distinguished sPCLs from pPCLs. Considering the entire dataset, almost all sPCLs were grouped with HMCLs, whereas most of pPCLs were in a separate cluster together with all MAF-translocated and the majority of t(4;14) MMs. Supervised analysis revealed 196 differentially expressed transcripts between pPCLs and sPCLs, all but one up-regulated in sPCLs. These transcripts are mostly involved in mitotic cell cycle, such as chromosome segregation and regulatory checkpoints, or in DNA recombination, replication and packaging. In particular, we found the up-regulation in sPCLs of several mitotic and transcriptional genes, whose coordinated and niche-dependent expression was associated with an increasing proliferation in MM. Moreover, we found the positively modulation of XRCC2, a gene involved in DNA recombination and reported to be linked to genomic instability in MM. The 196 genes were evaluated in the entire PC dataset, showing the strongest expression pattern in HMCLs. Additionally, 16 hsa-miRNAs resulted differentially expressed in PCL comparison. All of the up-regulated miRNAs in sPCLs belong to the miR-106a-363 (miR-18b, -20b, -92a, -106a) and miR-17-92 (miR-17, -18a, -20a, -92a) clusters, known to promote proliferation, angiogenesis and cell survival. Among the 8 down-regulated miRNAs in sPCLs, we found miR-152, whose down-regulation in hyperdiploid MM has been suggested to mimic the effect of translocations on specific oncogenic targets; and miR-29b, that negatively regulates migration and has been reported to play an anti-tumor activity in MM. *Conclusions.* The finding of specific gene and miRNA expression profiles in PCL may be useful to understand the molecular alterations discriminating the two forms of PC dyscrasia, thus providing a contribution to the identification of novel therapeutic targets.

C056

DNA COPY NUMBER AND WHOLE-EXOME SEQUENCING ANALYSES IN SEQUENTIAL MYELOMA SAMPLES AT DIAGNOSIS AND RELAPSE

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Introduction. Multiple myeloma (MM) is a plasma cell (PC) malignancy characterized by a marked genetic heterogeneity at onset, followed by further genomic complexity acquired during disease progression and particularly after treatment. To gain insight into the molecular evolution associated with MM progression, we investigated sequential samples of 7 MMs and 1 primary PC leukemia (pPCL) by genome-wide DNA copy number analysis and whole-exome sequencing (WES). *Methods.* Highly purified PC samples obtained at diagnosis and relapse after first line therapy (7 symptomatic MMs and 1 pPCL) were subjected to genome-wide DNA profiling (all cases) and WES (1 MM and 1 pPCL samples, with matched negative controls). Copy number data were generated on Affymetrix CytoScan HD Array, using the Chromosome Analysis Suite software. Single sample analysis was performed with default parameters and setting the Reference Model. WES was carried out on Illumina GAIIx platform and variant calling was performed using Mutect algorithm, by separately comparing primary and progression samples to its matched normal control. *Results.* Concerning regions of prognostic importance, 1p loss was identified as a novel lesion or evolving from a sub-clone in 3 relapsed samples, whereas 1q gain or 17p loss were respectively acquired in two cases. Notably, some alterations, present at diagnosis only in sub-clones, were detected in the majority of tumor cells in at least one of 5 relapsed MMs. Such lesions involved single or combined gains or losses of whole chromosomes (chr) 3, 8, 9, 18, 20, aberrations of short/long arms of chrs 11, 13, 14, 15, 18, 21 or smaller altered regions on chrs 4, 10, 12, 16, 17, 19. Interestingly, deletions involving chr 5q (3/8 relapsed MMs) or 8q (2/8 relapsed MMs) were detected as *de novo* acquired lesions. Furthermore, we investigated by WES two cases, a MM progressed to secondary PCL (sPCL) and a pPCL patient at diagnosis and relapse. Each patient showed a dynamic mutational pattern, involving both the acquisition and the loss of a large number of point mutations. Specifically, 19 genes were exclusively mutated in MM at diagnosis and 66 only in sPCL phase, whereas 12

genes were mutated in both conditions; in pPCL patient, 138 genes were evidenced at diagnosis and 166 at relapse, while 78 were commonly altered. Genes acquiring mutations in disease course were mostly involved in DNA repair, histone methylation, protein metabolism, regulation of NF-kB cascade, focal adhesion and MAPK signaling pathways. Concerning genes frequently altered in MM, it is worth reporting mutations of TP53 and CYLD at relapse, in sPCL and pPCL, respectively. *Conclusions.* Our data highlight the importance of using high-throughput approaches to provide insights into the definition of genetic alterations potentially related to mechanisms of drug resistance and MM progression.

Mesenchymal Stem Cells and Bone Marrow Microenvironment

C057

THE EXPRESSION OF HIGH LEVELS OF CRBN ISOFORM LACKING IMiDs BINDING DOMAIN PREDICTS FOR A LOWER PROBABILITY OF HIGH-QUALITY RESPONSE TO IMiD-BASED UPFRONT THERAPY IN NEWLY DIAGNOSED MULTIPLE MYELOMA PATIENTS

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In recent years, the immunomodulator derivatives (IMiDs) have been extensively used for the treatment of multiple myeloma (MM). The protein Cereblon, which is encoded by CRBN, located on chromosome 3, has been proposed as a primary target of IMiDs. In particular, in several studies the down-regulation of CRBN was linked to IMiDs resistance. Aims of this study were to set up and validate a consistent Real-time assay for the quantification of CRBN isoforms and to explore its role as biomarker for the response to IMiDs-based therapy. The study included a cohort of 100 patients (pts) with newly diagnosed MM who were homogeneously treated with IMiD- based induction therapy, prior to autologous stem cell transplantation. Response to therapy was evaluated according to the International Myeloma Working Group criteria. Gene expression profiling was performed according to standardized procedures (Affymetrix), using the HG-U133 Plus 2.0 array. The study was performed on the enriched CD138+ cell fraction obtained from pts' bone marrow samples collected at diagnosis. A Real-time assay was set up to get an absolute quantification of both the CRBN full-length isoform and the truncated one, lacking exons 8 and 10, which code for the IMiDs binding domain. The GAPDH gene was used to normalize expression data and final results were expressed as the percentage of the truncated isoform out of the whole amount of detected isoforms. The CRBN assay was tested on 100 newly diagnosed MM pts who were clustered in two subgroups according to the percentage of spliced CRBN (*e.g.* exceeding or not the threshold value of 4.49%). Group A included 34 pts who expressed >4.49% of spliced CRBN, while in group B were included 66 pts with a lower percentage of spliced CRBN. The two subgroups of pts were homogeneous for baseline clinical characteristics, except for a slightly higher frequency of t(4;14) in group B. After induction therapy, 23/34 (67.6%) group A pts failed at least a VGPR (very good partial response), whereas the remaining 10 achieved this objective (p<0,01, Fisher exact test). Conversely, group B pts the frequencies of pts achieving or failing at least VGPR were almost similar (46% and 54%, respectively). The differential expression of 1352 probe sets characterized the transcriptome profile of pts with higher amount of spliced CRBN: IRF4 resulted significantly down regulated in this subgroup of pts (FC=-1,304, p=0,004); the DNA damage control pathway resulted significantly de-regulated, as a consequence of the over-expression of CHK (FC=3,083, p=0,01), 14-3-3 (FC=2,266, p=0,02), CDC25 (FC=1,531, p=0,01) and the down-regulation of RAD9 (FC=-1,057, p=0,01). To conclude, we set up and validate a consistent Real-time assay for the quantification of actually available CRBN isoforms. By applying this assay, we found that a higher expression of spliced CRBN predicts for a lower probability to achieve a high-quality response to IMiD-based upfront therapy.

C058

ROLE OF STROMAL CELL-MEDIATED NOTCH SIGNALING IN B-CELL ACUTE AND CHRONIC LYMPHOPROLIFERATIVE DISEASES

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Introduction. Stromal cells are essential components of the bone marrow (BM) microenvironment regulating and supporting the survival of

different tumors, including B-cell acute and chronic lymphocytic leukemia (B-ALL and CLL). In this study, we investigated the role of Notch signaling in human BM-mesenchymal stromal cell (BM-MSC)-promoted ALL and CLL survival and chemoresistance. *Methods.* PCR, FACS analysis and western immunoblotting were used to study the expression of Notch receptors and ligands, as well as Notch activation, in B-ALL, CLL and BM-MSCs. Leukemic cells were co-cultured with BM-MSCs at 10/1 and 1/1 (B-ALL or CLL:BM-MSCs) ratios for 3, 7 or 14 days in presence of chemotherapeutic agents and in presence or absence of Notch-blocking antibodies or GSI-XII (Gamma Secretase Inhibitor-XII). Cell viability was evaluated by Annexin-V/PI; proliferation and cell cycle were assessed through CFSE dilution and Propidium Iodide, respectively. *Results.* The block of Notch signaling through GSI-XII reverted the protective effect mediated by co-culture with BM-MSCs. The treatment with combinations of anti-Notch neutralizing antibodies resulted in the decrease of B-ALL cell survival, either cultured alone or co-cultured in presence of BM-MSCs from normal donors and B-ALL patients. The inhibition of Notch-3 and -4 or Jagged-1/-2 and DLL-1 resulted in a dramatic increase of apoptotic B-ALL cells by 3 days, similar to what is obtained by blocking all Notch signaling with the GSI-XII. The same Notch receptors are involved in CLL survival except for Notch-1 that, in CLL, mediates a synergistic effect with other Notch receptors in inducing the anti-apoptotic phenotype. *Conclusions.* Overall, our findings show that stromal cell-mediated Notch signaling has a role in promoting ALL and CLL survival and resistance to chemotherapy. Therefore, the target of Notch pathway activation may represent a useful strategy to overcome drug resistance and improve the efficacy of conventional treatments.

C059

C-MYB RESTRAINS MEGAKARYOPOIESIS THROUGH THE HSA-MIR-486-3P-DRIVEN DOWN-REGULATION OF C-MAF

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Introduction. The transcription factor c-Myb plays a key role in human primary CD34+ hematopoietic progenitor cells (HPCs) lineage choice, by enhancing erythropoiesis at the expense of megakaryopoiesis. We previously demonstrated that c-Myb affects erythroid *versus* megakaryocyte lineage decision in part by transactivating KLF1 and LMO2 expression. To further unravel the molecular mechanisms through which c-myb affects lineage fate decision, we profiled the miRNA and mRNA changes in myb-silenced CD34+ HPCs. *Methods*. RNA from CD34+ HPCs transfected with c-myb-targeting/non targeting control synthetic siRNAs was collected 24 hours post-Nucleofection for a set of 5 independent experiments. mRNA and miRNA expression for each sample were profiled by Affymetrix HG-U219 array and Exiqon Human miRNome PCR Panel, respectively. miRNA/mRNA data were integrated by Ingenuity Pathway Analysis. The effects of hsa-miR-486-3p overexpression on CD34+ cells differentiation ability were studied by morphological and immunophenotypic analyses after liquid culture and by collagen-based clonogenic assay. Furthermore, gene expression changes in CD34+ cells upon hsa-miR-486-3p overexpression were profiled by Affymetrix HG-U219 array. *Results*. The integrative analysis of miRNA/mRNA expression changes upon c-myb silencing in human CD34+ HPCs highlighted 19 miRNAs with 150 anticorrelated putative target mRNAs. A set of miRNAs with the highest number of predicted target mRNAs was selected for functional studies. Among them, we identified hsa-miR-486-3p as a miRNA affecting CD34+ HPCs commitment. Indeed, morphological and flow cytometric analyses after liquid culture showed that hsa-miR-486-3p overexpression in CD34+ HPCs enhanced erythroid and granulocyte differentiation while restraining megakaryocyte and macrophage differentiation. Moreover, collagen-based clonogenic assay demonstrated a strong impairement megakaryocyte commitment upon hsa-miR-486-3p-overexpression in CD34+ cells. Gene expression profiling of hsa-miR-486-3p overexpressing CD34+ cells enabled us to identify a set of 8 genes down-regulated and computationally predicted, putative hsa-miR-486-3p targets. Among them, we selected c-maf transcript as up-regulated upon myb silencing. Worth of note, c-maf silencing in CD34+ progenitor cells was able to reverse the effects of myb silencing on erythroid *versus* megakaryocyte lineage choice. *Conclusions*. Integrative miRNA/mRNA analysis highlighted a set of miRNAs and anticorrelated putative target mRNAs modulated upon myb silencing, therefore potential players in myb-driven HPCs lineage choice. Among them, we demonstrated the hsa-miR-486-3p/c-maf pair as partially contributing to the effects of myb on HPCs commitment. Therefore, our data collectively identified c-myb-driven hsa-miR-486-3p up-regulation and subsequent c-maf down-regulation as a new molecular mechanism through which c-myb favours erythropoiesis while restraining megakaryopoiesis.

C060

INFLAMMATORY PATHWAYS IN THE BONE MARROW: ROLE OF TIMP-1 IN HEMATOPOIETIC STEM/PROGENITOR CELLS AND LEUKEMIA

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Introduction. Inflammation is a protective response that contains tissue injury and preserves homeostasis. However, a prolonged inflammation hinders tissue functionality and may play a role in cancer. Hematopoietic Stem and Progenitors Cells (HSPCs) reside within the Bone Marrow, a nurturing environment shielding HSPCs from external insults. However, recent findings showed that danger signals and inflammatory cytokines actively affect HSPCs. The Tissue Inhibitor of MetalloProteinases-1 (TIMP-1) is a member of the inflammatory network: first described as an inhibitor of MMPs, TIMP-1 also displays cytokine-like functions. We recently found that TIMP-1-/- mice have decreased BM cellularity and impaired engraftment capabilities due to cell-cycle defects. Aim of this study is to investigate TIMP-1's role in normal and leukemic hematopoiesis. *Methods.* Human CD34+ HSPCs were isolated from cord blood (CB) units, while leukemic cells were collected from AML patients at diagnosis. Cell proliferation was assessed by colony forming unit (CFU) assays, Long-Term Cultures (LTCs), cell cycle analyses, and CFSE staining. TIMP-1's contribution to cell survival was evaluated by AnnexinV/PI staining. Primitive HSC potential was assessed by transplantation of TIMP-1-treated CD34+ cells into immonodeficient mice (NOD/Shi-scid/IL-2Rγnull mice). Finally, the expression of the tetraspannin receptor CD63 (TIMP-1's putative receptor) was assessed by flow cytometry, whereas its functional role was tested by nucleofection of CD63-specific siRNAs in HSPCs. Downstream molecular targets of TIMP-1 (such as pAkt, CycD1, p27) were also evaluated by qPCR and flow cytometry. *Results.* rhTIMP-1 promotes CD34+ cell survival and stimulates HSPC expansion, without compromising the engraftment potential when transplanted into immunodeficient mice. The dissection of TIMP-1 signaling pathway indicated that the tetraspanin CD63 receptor is required for TIMP-1's cytokine functions in HSPCs. CD63 activation leads to PI3K recruitment and phopshorylation of AKT, key modulators of survival/proliferation pathways in HSCs. Downstream targets of pAKT are also modulated, including the proliferation marker CycD1, which levels are increased upon exposure to TIMP-1. In AML patients, preliminary findings indicate that, at diagnosis, TIMP-1 levels are increased in both the peripheral blood and the BM. Of note, TIMP-1 promotes AML cell maintenance *in vitro*, possibly due to anti-apoptotic effects. Similarly to CD34+ HSPCs, the colony-forming potential of primary human AML cells is also increased upon exposure to TIMP-1, suggesting a role of TIMP-1 in AML proliferation. *Conclusions.* Our study suggests that TIMP-1 may play a key role in the BM microenvironment, modulating both normal HSPCs and AML cell proliferation. Our findings have the potential to provide a novel therapeutic target for therapies targeting leukemogenic mechanisms sprouting from inflammatory microenvironments.

C061

GSK3β DOWN-REGULATION IN MESENCHYMAL STEM CELLS FROM PATIENTS WITH MYELODYSPLASTIC SYNDROME

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In addition to neoplastic transformation of hematopoietic progenitors, bone marrow microenvironment damages can contribute to

myeloid neoplasms development and maintenance. Several functional and morphological abnormalities of bone marrow mesenchymal stromal cells (BM-MSC) have been described in myeloid neoplasms. Nevertheless, molecular bases of differences between MSCs from normal and leukemic/myelodysplastic bone marrows are still unknown. Deregulation of genes belonging to PI3K/AKT signaling pathway has been described in MSC from different type of cancers. We studied the expression profile of genes belonging to PI3K/AKT signaling pathway in MSCs from 40 patients including 10 *de novo* AML, 10 *de novo* MDS, 10 t-MN (therapy-related myeloid neoplasms) and 10 patients with limited stage lymphoma without bone marrow involvement (used as normal control). BM-MSCs were obtained by Ficoll-gradient centrifugation of bone marrow samples and cultured up to 70% confluence in MesenCult Medium. Cells at 2nd passage were used for all experiments. The Human PI3K-AKT PCR array (SABioscience) was used to analyze mRNA levels of 84 key genes involved in PI3K-AKT signaling pathway, comparing 5 *de novo* AML, 5 *de novo* MDS and 5 t-MN *vs* 5 normal bone marrows. Relative changes in gene expression were calculated using the ΔΔCt method. Fold change variations ≥1.5 in association to statistically significant T-test (p-value≤ 0.05) were used for the statistical analysis. Genes resulted significantly deregulated were validated by quantitative real time RT-PCR, in 10 *de novo* AML samples, 10 *de novo* MDS, 10 t-MN and 10 normal controls. Total GSK3β protein level and its Ser-9 phosphorylated isoform were measured by Western blot in a subgroup of MDS patients and controls. PI3K-AKT PCR arrays revealed a significantly down-regulation of GSK3β, MTCP1, RASA1 and SOS1 genes in MSC from all leukemic and myelodysplastic bone marrows compared to control group. After validation, all genes were confirmed as significantly down-regulated in *de novo* MDS samples respect to normal controls: GSK3β (p=0.0056, FC=-1.94), RASA1 (p =0.0044, FC=-2.19), SOS1 (p=0.0047, FC=-1.9) and MTCP1 (p=0.0026, FC=-1.93). No significant differences were found in the expression levels of studied genes in the validation group among *de novo* AML, t-MN and controls. Western blot analysis confirmed the down-regulation of both GSK3β total protein and of its Ser-9 phosphorylated isoform in MDS MSC respect to controls. Deregulation of genes belonging to PI3K/AKT signaling pathway may contribute to MSC dysfunction described in MDS bone marrows and can affect their ability to interact with normal hematopoietic cells, participating to bone marrow failure and myelodysplastic development. GSK3β, a crucial regulatory kinase interacting with multiple signaling pathways, is one of the most significantly down-regulated genes in MSC from myelodysplastic bone marrows and its functional significance is under investigation.

C062

KNOCKOUT OF CSNK2β DURING HEMATOPOIESIS RESULTS IN MID/LATE GESTATION LETHALITY MAINLY DUE TO IMPAIRED FOETAL ERYTHROPOIESIS

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Introduction. CK2 is a serine-threonine kinase composed of two catalytic (α) and two regulatory (β) subunits and its involvement in hematological malignancies has been recently investigated. This kinase regulates the PTEN/PI3K/AKT, Wnt/βcatenin, Hedgehog, Jak-Stat and NF-κB signalling cascades, all of which are critical for stem cell biology and normal hematopoiesis. However, the role played by CK2 during blood cell development has remained as yet unexplored. *Methods.* Here, we sought to examine the function of CK2 in hematopoiesis by generating conditional knockout mice for Csnk2B crossing Csnk2β-Flox/Flox mice with Vav1-CRE transgenic mice to inactivate Csnk2β starting from 9.5 dpc during embryonic development. Histo-cytological methods, Flow Cytometry, colony forming assays, signal transduciotn analysis by wetern blotting and RT-PCR were employed. *Results.* Only rare pups were found at birth. Csnk2β knockout in the hematopoietic system resulted in in utero lethality at mid-late gestation. Macroscopic and phenotypic analysis during gestation revealed the appearance of pale and hydropic fetuses after 12.5 dpc. Some pups showed haemorrhages. Fetal livers appeared smaller and paler. Cytological analysis and colony forming assay studies unveiled a great depletion of hematopoietic elements belonging to both the erythroid, megakaryocytic and myeloid precursors. A more thorough analysis of the erythroid phenotype revealed that Csnk2β loss caused red cell impairment/loss at two developmental stages: at the earlier stages of Megakaryocyte-Erythroid Precursors (MEP) and proerythroblasts and at later stages of terminal erythroid maturation (orthocromatic erythroblasts, reticulocytes). A critical role for CK2 in terminal erythroid differentiation was also found in *in vitro* differentiation assays using hemin and the CML cell line K562. Western blot and RT-PCR analysis highlighted perturbations in cell cycle regulatory proteins as well as cellular apoptosis in Csnk2b knockout liver cells. In particular, Csnk2β loss was associated to a marked reduction of total and phosphorylated Akt and a decrease of Hedgehog target genes such as Gli-1 and Cyclin D1. Moreover, starting form 14.5 dpc, liver cells displayed a massive p53-dependent response, leading to high levels of p21. *Conclusions.* Our work establishes a critical role for Csnk2β in haematopoiesis that will be further characterized in all its components.

C063

OVEREXPRESSION OF CDKN2B IS ASSOCIATED WITH REDUCED PROLIFERATIVE POTENTIAL IN MESENCHYMAL STEM CELL FROM HIGH-RISK MYELODYSPLASTIC SYNDROME

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Introduction. Myelodysplastic syndromes (MDSs) are a heterogeneous group of clonal hematopoietic stem cell (HSC) malignancies that are characterized by ineffective bone marrow hematopoiesis, peripheral blood cytopenias, and a substantial risk for progression to acute myeloid leukemia. There is evidence that alterations of bone marrow stromal cells contribute to abnormal HSC growth and maturation. *Methods.* Here, we characterized the structural and functional properties of bone marrow-derived mesenchymal stem cell (BM-MSCs) isolated from 24 high-risk IPSS MDS patients studying their cell cycle activity, gene expression profile and mechanisms involved in replicative senescence. These cells were also studied for their functional properties, including their hematopoietic supporting role and global DNA methylation status. *Results.* MDS-MSCs achieved confluence at a significantly lower rate than donors and displayed reduced proliferative capacity. In particular 40% of samples were unable to expand. To study the long-term hematopoietic mantaining ability, MDS-MSCs were culture with CD133+ cells, showing a decreased ability to support the growth of myeloid and erythroid progenitors (p<0.05). This reduced proliferative capacity suggested changes in the cell cycle activity. Therefore, we studied the gene expression profiles for 37 regulatory genes observing CDKN2B up-regulation in MDS-MSCs (8 times higher than donors). These data might be associated with premature cellular senescence but no up-regulation of CDKN1A and CDKN2A, other typical senescence markers, was observed. Moreover, no significant differences between MDS-MSCs and donors in the beta-galactosidase expression, telomere length, apoptosis and satellite 2 methylation status were found. No genomic instability was observed. Furthermore MDS-MSCs exhibited globally hypermethylated DNA that was 2.2 times higher than donors even if no difference in the levels of DNMT1, DNMT3a and DNMT3b mRNA was observed. *Conclusions*. In conclusion, we showed that MDS-MSCs were structurally, epigenetically and functionally altered suggesting that MSCs could play a critical role in MDS pathogenesis and could be a possible target for new therapeutic strategies.

C064

THE INCREASED PLATELET APOPTOSIS OF IMMUNE THROMBOCYTOPENYA IS DUE TO A DEREGULATED INTRINSICALLY PROGRAMMED CELL DEATH AND IS TRIGGERED BY (AUTO)ANTIBODIES LIGATION OF PLATELET SURFACE GLYCOPROTEIN RECEPTORS

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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 dic-

tates 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. Thus, platelets are by default genetically programmed to die by apoptosis and the antagonistic balance between Bcl-xL and Bak constitutes a molecular clock that determines platelet life span (Mason, 2007). In addition, antibodies against platelet surface glycoproteins (GPs) participate in the triggering of apoptosis in mouse platelets (Leytin, 2006). Here, we investigated whether and to what extent the intrinsic pathway of platelet apoptosis is deregulated and whether antibodies-targeted GP play a role in mediating apoptosis in human Immune Thrombocytopenia (ITP). *Methods.* We studied 30 patients with active chronic ITP. Flow cytometry analysis of apoptosis and Bcl-xL/Bak proteins expression were performed on freshly isolated and *in vitro* aged platelets in the presence of autologous/allogeneic platelet poor plasma (PPP) from healthy individuals and ITP patients with (Ab+)/without antiplatelet antibodies (Ab-) or in the presence of monoclonal antibodies (MoAb) directed against platelet GPIIb (CD41) and GPIb (CD42b). PS exposure induced by ABT-737, a selective Bcl-xL-inhibitor, was also investigated. *Results.* We found that in ITP, together with increased PS exposure, Bcl-xL protein, but not Bak, shows reduced expression and susceptibility to the ABT-737-induced inhibition in *in vitro* aged platelets. Interestingly, Bcl-xL protein expression of *in vitro* aged platelets from Ab+ ITP patients is significantly reduced as compared with that of platelets from Ab- ITP patients. Consistently, PPP from Ab+ ITP patients, but not that from Ab- ITP or from healthy subjects, significantly increases the PS exposure of normal platelets. In addition, at variance with CD41 MoAb, normal or ITP platelets show increased PS exposure after *in vitro* incubation with anti-CD42b MoAb. However, Bcl-xL protein expression was not reduced after *in vitro* incubation of normal platelets with PPP from Ab+ ITP patients or with anti-CD42b MoAb. *Conclusions.* We demonstrate that in ITP increased platelet apoptosis is due to a deregulated intrinsically programmed cell death and may be triggered by (auto)antibodies ligation of platelet GPs. Therefore, in addition to the classical pathway of IgG-coated platelets phagocytosis, a novel mechanism of PS-labelled platelets clearance may be suggested to trigger and/or to worsen thrombocytopenia in ITP.

Miscellaneous

C065

PREVALENCE OF COMBINATION OF TRIPLE ALLELIC MUTATIONS ASSOCIATED WITH THROMBOPHILIA IN PATIENTS WITH VENOUS THROMBOEMBOLISM

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Introduction. Triple thrombophilic abnormalities have been rarely reported and most patients carried polymorphisms nowadays not included in the recommended laboratory investigation for thrombophilia, such as those in the MTHFR gene. Aim of the study is to assess the prevalence of triple allelic mutations associated with thrombophilia in patients with venous thromboembolism (VTE). *Methods.* We selected from a cohort of 2,199 individuals with VTE who were referred to our Thrombosis Center those with triple thrombophilic abnormalities (*i.e.* deficiency of antithrombin [AT], protein C [PC], protein S [PS], factor V Leiden [FVL], prothrombin [PT] 20210A) and/or triple allelic mutations (*i.e.* triple heterozygosity [Het], homozygosity [Hom] plus Het). *Results.* We identified 10 patients (M/F 5/5) (0,45%) (Table 1). Four patients had triple Het, and the remaining carried a combination of Hom and single Het. No patient in the cohort carried double Hom neither Hom for deficiency of natural anticoagulants, nor triple deficiency of natural anticoagulants. Het for deficiency of natural anticoagulants was present in 5 patients (AT=2, PC=1, PS=1, PC+PS=1), associated with FVL or PT20210A (Table). Four patients had triple abnormalities (various combinations of AT,PC,PS deficiency, and FVL or PT20210A) (0.18%), and 6 had double abnormalities (AT deficiency and Hom for FVL in one case, FVL and PT20210A in the remaining ones). The first VTE was deep vein thrombosis (DVT) of one leg in 9 cases, in 2 of them with pulmonary embolism (PE), and superficial vein thrombosis (SVT) in 1 case. The median age of first VTE was 27 years (range 2-73), in 8 cases <45 years. The first event was provoked in all cases. Two of them had an additional thrombophilic acquired abnormality (hyperhomocysteinemia and lupus anticoagulant, respectively). Four patients had recurrent VTE events. Eight patients received lifelong treatment with vitamin K antagonists (VKA). *Conclusions.* In patients with VTE triple allelic mutations associated with thrombophilia are uncommon but not exceedingly rare (0.45%), and diagnosis of a single thrombophilia abnormality should not discourage from an exhaustive laboratory investigation. Abnormality can be a combination of Hom and Het or a combination of triple Het. The clinical onset occurs in young age in the large majority of cases. Surprisingly, such conditions seem not associated with unprovoked events, and recurrence occur in a minority of subjects.

Table 1.

Patient	Sex	Genotype	Family history	Age of onset	First VTE	Risk factors	Recurrence	VKA
P.M.	F	Triple Hetero (AT+ FVL+ FIIA)	Yes	2	DVT	Viral infection (measles)	Yes	Ongoing
G.B.	M	Triple Hetero (PC+ FVL+ FIIA)	Yes	41	DVT+PE	Surgery	Yes	Ongoing
L.G.	F	Triple Hetero (PS+ FVL+ FIIA)	Yes	73	DVT	Pneumonia	No	Ongoing
A.R.	F	Triple Hetero (PC+ PS+ FIIA)	Yes	28	DVT	Oral contraception	Yes	Ongoing
C.P.	M	Hetero AT + Homo FVL	Yes	57	DVT	Obesity Bed rest	No	No
E.D.	M	Homo FVL + Hetero FIIA	Yes	26	DVT	Viral infection (mononucleosis) Bed rest	No	Ongoing
M.D.	F	Homo FVL + Hetero FIIA	Yes	26	DVT+PE	Oral contraception HyO	No	No
V.O.	M	Homo FVL + Hetero FIIA	Yes	16	DVT	Trauma	No	Ongoing
G.G.	F	Hetero FVL Homo FIIA	No	24	DVT	Puerperium	No	Ongoing
G.S.	M	Hetero FVL Homo FIIA	No	28	SVT	Trauma Lupus anticoag.	Yes	Ongoing

C066

ENERGY RESTRICTION REGULATES BCR/ABL EXPRESSION IN CHRONIC MYELOID LEUKAEMIA CELLS VIA TRANSCRIPTIONAL/POST-TRANSCRIPTIONAL CONTROL

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Introduction. We previously demonstrated that severe hypoxia selects Leukaemia Stem Cells (LSC) of Chronic Myeloid Leukaemia (CML) and that BCR/Abl protein is suppressed in hypoxia-selected CML cells. Thus, LSC are independent of BCR/Abl signalling and thereby insensitive to Imatinib-Mesylate (IM). The aim of this work is the characterization of the regulatory effects of energy restriction (hypoxia or glucose shortage) on BCR/Abl expression and CML cell growth. *Methods*. The human BCR/Abl-positive K562 and KCL22 CML cell lines are cultured with or without D-glucose, in air or in hypoxia (0.1% oxygen in the incubation atmosphere). BCR/Abl expression is determined by Western blotting (protein) or Real-Time PCR (mRNA); mRNA and protein stability is determined after treatment with Actinomycin-D or Cycloheximide, respectively; the level of activation of BCR promoter in hypoxia with respect to normoxia is assessed by ChIP and via the transient transfection of CML cells with a luciferase reporter vector carrying the BCR promoter. Post-transcriptional, translational and post-translational BCR/Abl regulation are evaluated by determining the polysome profile (sucrose gradient), immunofluorescence for RNA binding proteins (AUBP) and treatment with proteasome inhibitors. *Results*. Our results indicate that BCR/Abl protein suppression is actually determined when glucose shortage complicates the effects of low oxygen, indicating that ischemia-like conditions drive LSC refractoriness to IM. Oxygen shortage reduces BCR/abl mRNA levels (and possibly half-life), while glucose shortage does not, although either condition suppresses BCR/Abl protein. Accordingly, both hypoxia and glucose shortage reduce CML cell population expansion. On the other hand, BCR/Abl protein half-life is reduced under glucose, but not oxygen, shortage. Thus, BCR/Abl expression seems to be regulated differently within the two different niche environments. ChIP and luminescence assays (transcriptional regulation; hypoxia), polysome profile analysis and AUBP involvement (post-transcriptional/translational regulation; hypoxia or glucose shortage) and experiments with proteasome inhibitors (post-translational regulation; hypoxia or glucose shortage) are in progress. *Conclusions*. The results of this study suggest that BCR/Abl protein suppression is a distinctive feature of CML cells adapted to energy shortage. This suppression heavily affects the natural history of disease, as it represents the crucial condition for the selection of BCR/Abl(protein)-negative LSC of CML. Preliminary data suggest that glucose shortage is less efficient than oxygen shortage in selecting LSC. This might reflect a different "deepness" of BCR/Abl suppression in the two cases, as well as the adaptation of LSC to different microenvironments. These findings provide an insights into the molecular mechanisms of BCR/Abl regulation which may be relevant to the induction of primary resistance (refractoriness) of CML cells to therapy.

C067

ASSOCIATION OF AZACITIDINE AND LENALIDOMIDE (COMBINATION VS SEQUENTIAL TREATMENT) FOR HIGH-RISK MYELODYSPLASTIC SYNDROMES (IPSS RISK: HIGH OR INT-2): A PHASE II STUDY

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Introduction. Azacitidine (AZA) is able to induce hematologic responses in 50-60 % of patients (pts) with Myelodysplastic Syndromes (MDS) and moreover to prolong survival in higher risk MDS pts. However the duration of therapeutic response to AZA is limited, with a median survival advantage of only 9.5 months. Recently, several studies have evaluated the efficacy and safety of combining, in high-risk MDS pts, AZA with Lenalidomide (LEN), either administered concurrently (Sekeres, 2010; 2012), or sequentially (Platzbecker, 2013), in both cases showing promising results. The aim of this study was to evaluate the efficacy (ORR) and safety of the combination *vs* the sequential use of AZA and LEN in high-risk MDS pts. *Methods.* This is a randomized, phase II, multicenter, open label study, including pts with MDS (according to WHO 2008 classification) with International Prognostic Scoring System (IPSS) risk High or Intermediate-2, without previous treatment with AZA or LEN. ARM 1 (combined treatment): AZA: 75 mg/m2/day (days 1-5) I.C. + LEN: 10 mg/day (days 1-21), orally, every 4 weeks. ARM 2 (sequential treatment): AZA: 75 mg/m2/day (days 1-5) I.C. + LEN: 10 mg/day (days 6-21), orally, every 4 weeks. The treatment for both arms was planned for 8 cycles (32 weeks) in the absence of disease progression or unacceptable toxicity. A sample size of 44 pts was planned. *Results.* From March 2013, 37 pts (21 males), with a median age of 72 (48-83 yrs) were enrolled, from 13 hematologic italian Centers. At baseline, WHO diagnosis was: Refractory Cytopenia with Multilineage Dysplasia (RCMD): 3 pts; Refractory Anemia with Excess of Blasts-1 (RAEB-1): 9 pts; RAEB-2: 23 pts; MDS-unclassified (MDS-U): 2 pts; IPSS risk was: Intermediate-2: 29 pts; High: 6 pts; not determined (N.D.) (because of lack of cytogenetic data): 2 pts. 17 pts were randomly assigned to ARM 1, and 20 pts to ARM 2. At the time of this analysis, 19/37 pts (51.3%) completed ≥ 6 cycles of treatment, and are evaluable for response. 11/19 pts (57.9%) showed a favourable response to treatment, following the International Working Group (IWG) criteria (Cheson, 2006): 2 pts achieved Complete Remission (CR), 2 pts attained Partial Remission (PR), and 7 pts showed Hematological Improvement (HI), while the 8 non responder pts maintained a Stable Disease (SD). Responder pts were 7/8 (87.5%) in ARM 1, and 4/11 (36.4%) in ARM 2, respectively. Median time to response: 2 (2-7) months. A significant toxicity (grade > 2) was observed in 10/37 (27%) pts. 15/37 pts (40.5%) had a dose reduction of LEN because of hematologic or non-hematologic toxicity. *Conclusions.* Our results, although preliminary, seem to confirm the feasibility of the association of AZA and LEN in high-risk MDS pts. More data are needed in order to compare the efficacy and safety of combined *vs* sequential treatment. Moreover, a possible relationship with signal transduction pathways will be evaluated.

C068

DIFFERENTIAL EFFECT OF LENALIDOMIDE ON INDUCTION OF INOSITIDE-DEPENDENT ERYTHROPOIESIS AND CELL CYCLE IN MYELODYSPLASTIC SYNDROMES

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Introduction. Erythropoiesis is associated with the activation of specific inositide-dependent signalling pathways, such as Akt/PLCgamma1. On the other hand, Akt can also be associated with cell cycle and differentiation in MDS, via activation of mTOR pathway. Lenalidomide is currently used in the treatment of del(5q) low-risk MDS patients, to compensate and counteract the MDS ineffective erythropoiesis and selectively inhibit cell cycle in del(5q) cells. The exact molecular mechanisms underlying the effect of Lenalidomide in del(5q) and non-del(5q) MDS cells are still unclear, even though it is clear that Leanlidomide can target signalling molecules playing a role in the maintenance of the balance between apoptosis, proliferation and differentiation, such as Akt. *Methods.* We studied 6 patients diagnosed with del(5q) Low-Risk MDS (IPSS: Low or Int-1) who were given Lenalidomide. We also analyzed the effect of Lenalidomide on two cell lines: Namalwa CSN.70, bearing a del(5q) karyotype, and U937, with a normal 5q chromosome. In particular, we quantified the expression of several genes implicated in inositide signalling, such as Akt, mTOR and PI-PLCgamma1, as well as Cyclins and Globin genes, in order to assess the effect of Lenalidomide on cell cycle and erythropoiesis. *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 a specific phosphorylation of Akt only in non-del(5q) cells, as assessed by co-localization experiments. 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. As for cell lines, our findings hint at a specific activation of cell cycle and erythropoiesis only in U937 cells, whereas cell cycle, but not erythropoiesis, was selectively inhibited in Namalwa CSN.70 cell line, so that in these cells proliferation is slower than in non-del(5q) cells. *Conclusions.* In our case series, as well as in cell lines, erythropoiesis activation is associated with a response to Lenalidomide, with an induction of Akt/PLCgamma1. Moreover, only in non-del(5q) cells, a normal proliferation is allowed, given that in del(5q) cells our analyses show a cell cycle arrest and an inhibition of Akt/mTOR pathway. Therefore, our data support the hypothesis of a specific activation of both inositide-dependent proliferation and erythroid differentiation pathways in response to Lenalidomide treatment in non-del(5q) cells, whereas in the del(5q) cell clone there is a cell cycle arrest and a slower erythroid differentiation. Taken together, these results point to a specific activation of signalling pathways during Lenalidomide administration and possibly pave the way to a larger investigation aiming to assess the role of these pathways during the therapy.

C069

COMBINED UNFAVOURABLE POLYMORPHISMS OF hOCT1 AND ABCG2 ARE RESPONSIBLE FOR A DELAYED COMPLETE CYTOGENETIC RESPONSE DURING IMATINIB TREATMENT

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In the recent years, imatinib has changed the outcome of patients affected by chronic myeloid leukemia (CML); nevertheless, about one third of patients must stop the treatment for side effects or resistance. In both these contexts, the adherence to treatment and the drug plasma levels have been reported to be strictly linked both to the quality of response and to the tolerability. A large work on Imatinib pharmacokinetics is at present carried out according to the procedures of the TIKlet protocol (ClinicalTrials.gov identifier: NCT 01860456), thus extending the work previously published by our group (Di Paolo *et al.,* Pharmacogenomic J, 2014) on the role of several genetic covariates. As additional side study, we have investigated the possibility that the time to the achievement of the complete cytogenetic response (CCyR) could be influenced by the genetic polymorphisms in the trans-membrane transporters, such as SLC22A1, ABCB1, ABCG2, hOCT1, and SLCO1B1. To accomplish with this objective, both logistic regressions and Anova tests were applied in a population composed by 43 adult CML patients on imatinib. The goal of our approach was to investigate the possible role on CCyR of single and combined different genetic polymorphisms. From a first preliminary check we have found that the only significant role is played by the polymorphisms combination of ABCG2 and hOCT1. Through logistic regression we have computed the probability of obtaining a delayed CCyR (lasting more than 12 months) as a function of ABCG2 and hOCT1. We found that patients with favourable genotype (high-activity wild-type hOCT1 and low-activity polymorphic ABCG2) have a high probability of achieving the CCyR by 6 months of treatment, while patients with unfavourable genotype (both high-activity wild-type hOCT1 and ABCG2, or both low-activity polymorphic hOCT1 and ABCG2) have a significantly higher probability of achieving a late CCyR (>12 months). The logistic regression performed can be considered significant since patients with an unfavourable genotype have an Odd Ratio (O.R.) value equal to 1.15, p=0.002. Subsequently Anova tests have confirmed these results. In conclusion, our study demonstrated that a delayed cytogenetic response is influenced by the combination of the genetic polymorphisms that concomitantly cause a reduced intake (hOCT1) and an increased efflux (ABCG2) of imatinib, whereas there is not any observed effect due to each single polymorphism. This could be relevant, considering that the 2013 ELN guidelines identify as optimal responder patients who achieve the CCyR by 6 months from the beginning of therapy.

C070

INVESTIGATING CYTOPENIA MAY REVEAL BONE MARROW METASTASIS: A TEN-YEAR SINGLE CENTER HEMATOPATHOLOGY EXPERIENCE

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Introduction. The involvement of bone marrow (BM) by non-hematopoietic neoplasms usually occurs as a late event during the course of oncologic diseases. Most patients with BM metastases have hematological abnormalities, such as one or more cytopenias. BM biopsy is a key diagnostic tool to investigate unexplained persistent cytopenias. Limited information is reported in the literature about the incidence of BM metastasis during the course of solid malignancies, with considerable variability depending on the different types of neoplasms. *Methods*. A total of 9975 BM trephine biopsies were examined at our Hematopathology Laboratory, from January 2004 to December 2013. After processing, all samples were stained with Hematoxylin-Eosin (H&E), Giemsa and Silver impregnation (Gomori reaction). After morphologic evaluation, the appropriate immunohistochemical panel, including cytokeratin (CK) mix (AE1-AE3), was performed. *Results*. Solid cancer metastasis was documented in 95 of all 9975 BM biopsy samples (0.95%). Median patient age was 68 years (range 7-88 years; excluding a pediatric case, the interval span was 37-88 years). Males and females were 43 (45%) and 52 (55%) of the 95 cases, respectively. The most frequent metastatic tumors encountered were, in order of frequency, as follows: breast (40%), gastrointestinal (18%), lung (13%) and prostate (13%) carcinomas (Figure 1 A). In 9 cases (0.09%) a previous diagnosis of solid cancer was missing. All of them were of epithelial origin, according to the positive staining for CK mix. Primary tumor site was determined in 3 out of 9 cases (33.3%). In one case, estrogenic and progesterone receptors positivity revealed breast origin, despite the absence of radiological findings. Another case was of renal origin, considering a kidney mass found on CT-scan and MNF-116 positivity. The last case was diagnosed as gastric adenocarcinoma, confirmed by stomach biopsy. In the remaining 6 cases (66.6%), the rapid deterioration of clinical conditions contraindicated further diagnostic investigations. The most common hematological finding was anemia (79%), followed by thrombocytopenia (65%), and more rarely, by either leucopenia (24%) or leukocytosis (9%) (Figure 2 B). The finding of BM metastases predicted unfavorable outcome, with a median survival after diagnosis of 7 weeks (range 0-236 weeks).

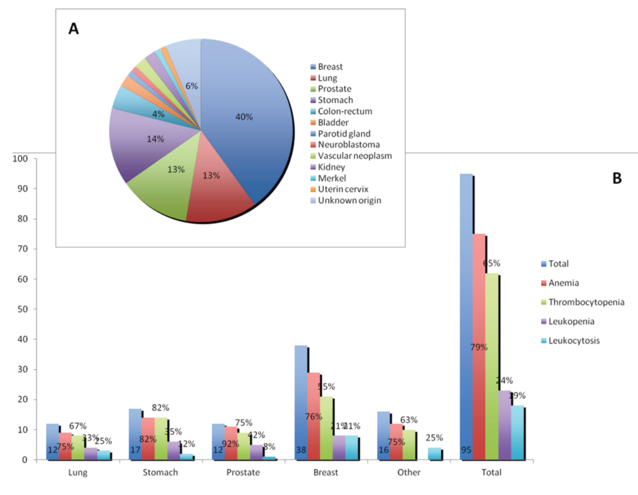

Figure 1.

Conclusions. BM biopsy is an invasive procedure mainly used for the diagnosis of hematopoietic disorders, but a significant proportion of biopsies are also performed to complete staging of non-hematopoietic tumors, at either diagnosis or disease progression. Involvement of the BM by non-hematopoietic lesions can incidentally be observed. Our case series highlighted that persistent unexplained cytopenias in solid cancer patients can be the first indicator of BM metastases. Although rarely, the finding of BM metastasis can lead to the first diagnosis of solid organ malignancies.

C071

CONSENSUS PAPER ON: "PLATELET CUT-OFF FOR ANTICOAGULANT THERAPY IN CANCER PATIENTS WITH VENOUS THROMBOEMBOLISM AND THROMBOCYTOPENIA"

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Introduction. Cancer-related Venous Thromboembolism (VTE) requires treatment with Low Molecular Weight Heparin (LMWH), which is more effective and safer than warfarin; however, the risk of major hemorrhage still remains clinically relevant (up to 5%). This rate is even higher in case of impaired hemostasis (due to myelosuppression or chemo-therapy), such as it may occur during thrombocytopenia. At the present, no guidelines are available regarding the best management of patients with acute or non-acute cancer-related VTE during thrombocytopenia. Aims: To develop a consensus about the platelet cut-off for safely administering LMWH in cancer patients with acute (=0r< then 1 month) and non-acute VTE and thrombocytopenia, based on RAND/UCLA Appropriateness Method (RAM). *Methods.* A systematic review of the literature was performed. Topics were cancer, venous thromboembolism, platelets, risk of bleeding, anticoagulant drugs, low-molecular-weight heparin and treatments. A questionnaire of appropriateness of the use of different doses of LMWH, according to the platelet count, was produced. A panel of experts was identified; the literature review and the list of indications were sent to all panel members. For each indication, the experts rate the benefit-to-harm ratio of the procedure on a scale of 1 to 9. *Results.* The panel reached the following consensus: Appropriate:- In acute VTE (including catheter-related VTE): Full dose of LMWH if PLT >50,000 <100,000/ mm3 (9/9); Reduced dose to 50% of the full dose, if PLT > 30,000 < 50,000/mm3 ; The discontinuation of LMWH treatment if PLT < 30,000/mm3 (6/9); and, in case of DVT of the lower limbs, the positioning of IVC filter (6/9). -In non-acute VTE (including catheter-related VTE): LMWH reduced to 75% of full dose, if PLT > 50,000 <100,000/mm3 (7/9); The discontinuation of LMWH treatment if PLT< 30,000/mm3 (7/9). Uncertain:- In acute VTE: Full dose of LMWH if PLT > 30,000 < 50,000/ mm3 (7/9). - In non-acute VTE (including catheter-related VTE): LMWH reduced to 75% of full dosage if PLT>30,000<50,000/mm3(7/9); LMWH discontinuation and insertion of IVC (in case of DVT), if platelets < 30,000/mm3(5/9); LMWH reduced to 75% of full dosage if PLT >30,000<50,000/mm3(6/9); Inappropriate:- In acute VTE: Full dose of LMWH if platelets<30,000 mm3 (9/9); The discontinuation of LMWH if PLT> 30,000<50,000/mm3(6/9). -In non-acute VTE (including catheter-related VTE): Reduced dose to 75% LMWH if PLT<30,000/mm3(7/9); The discontinuation of LMWH if PLT>30,000<50,000/mm3(5/9). Summary/*Conclusions.* This is the first expert opinion based on RAM to establish the safe platelet cut-off to administer LMWH therapy in patients affected by acute and non-acute VTE. The panel suggest as appropriate the use of dose-adjusted LMWH according to platelets count. Further investigations by means of well-designed prospective clinical trials are needed to establish the best management of cancer-related VTE in patients with thrombocytopenia.

C072

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

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Introduction. Ferroportin (FPN) is the only known iron exporter protein. It is expressed in different cell types including macrophages and erythroblast. Recently it has been reported the existence of different FPN transcripts with or without an iron-responsive element (IRE) in their promoter (FPN1A and FPN1B). The expression of these isoforms and the mechanisms regulating their expression in erythroid cells in non transfusion-dependent beta thalassemia syndromes (NTDT) are not known. *Aims.* To investigate the expression profile of FPN isoforms during erythroid differentiation in control and NTDT cell cultures and to elucidate their mechanisms of expression. *Methods.* An *in vitro* model of erythropoiesis derived from human peripheral CD34+ cells from healthy volunteers (control) and NTDT patients was used. The expression pattern of FPN isoforms was evaluated at day 0, 7 and 14 of culture by real-time PCR (2^-dCt). The relative percentage of each isoform was calculated on the basis of total FPN expression (FPN1A+FPN1B).The intracellular iron concentration was analyzed using an Iron Assay Kit (Biovision). In independent experiments, control cultures were treated with Ferric Ammonium Citrate (FAC), heme (Hemin) or hydrogen peroxide (H2O2) to investigate a possible role of these compounds in FPN regulation. FPN expression was evaluated at day 14 in standard and treated conditions by real-time PCR (2^-ddCt; untreated cells used as calibrator). *Results*. The FPN expression increased during erythroid differentiation, with the highest level at day 14 both in control and NTDT cultures (Table 1). The FPN1A was the more expressed isoform in both conditions.Its expression was higher at day 0 and 14, while FPN1B expression was higher at day 7. Noteworthy, the FPN1B expression, although lower compared to FPN1A, was significantly higher in NTDT cultures than in control ones, particularly at day 14. The intracellular iron concentration decreased significantly from day 7 to day 14, in control and NTDT cultures; however, at day 7 the iron levels in NTDT cultures were notably lower than in controls. The addition of FAC and Hemin in control cultures did not modified the FPN expression compared to untreated cultures; H2O2 added to control cells increased the expression of both FPN isoforms (Table 2.). *Conclusions*. The FPN expression increases during erythroid differentiation in control and in NTDT cultures, suggesting its role in exporting the excess intracellular iron. In both conditions the FPN1A is the more expressed isoform. However the expression of the non-iron responsive FPN1B isoform, altough lower compared to FPN1A, is significantly higher in NTDT than in control conditions. In control cultures, FPN expression, and particularly the FPN1B isoform, seems to be regulated by H2O2. These data suggest that the oxidative stress, higher in NTDT conditions, could be one of the major regulator of FPN1B expression, with a major iron export from NTDT erythroblast cells.

Table 1.

		Day 0	Day 7	Day 14
Control	Total FPN	0,16 ± 0,11	0,15 ± 0,08	2,3 ± 1 *
	FPN1A%	95 ± 2	70 ± 4	97 ± 1,7*
	FPN1B %	5 ± 2	30 ± 4•	3 ± 0,7
	intracellular IRON (nM)	n.d.	28 ± 2,8	9,5 ± 4,5*
NTDT	Total FPN	0,4 ± 0,17	0,14 ± 0,04	2,9 ± 1,7*
	FPN1A%	93 ± 2,5	58 ± 8	95 ± 2,5*
	FPN1B %	7 ± 2,5	42 ± 8•	5 ± 2,5°
	intracellular IRON (nM)	n.d.	13,5 ± 3,6°	5,8 ± 3,4*

• day 7 vs day 0, * day 14 vs day 7, ° NTDT vs Control: $p< 0.05$

Table 2.

	Untreated	FAC100uM	Hemin 10uM	H_2O_2 0,1mM
FPN1A	1	0.9 ± 0.5	0.9 ± 0.01	1,73
FPN1B	1	0.9 ± 0.2	1 ± 0.01	2,11

Immunoregulation and Immunotherapy

C073

A SUSTAINED CD16 AGGREGATION INDUCED BY THERAPEUTIC ANTIBODY-OPSONISED TARGETS IMPAIRS CYTOTOXIC RESPONSES IN HUMAN NK CELLS

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Purpose. It is well recognised that the efficacy of anti-CD20 antibody (Ab)-based immunotherapeutic strategies largely relies on the ability to induce ADCC by means of the aggregation of the FcgammaRIIIA, CD16, on NK cells. We address the impact of receptor aggregation by anti-CD20 Ab-opsonised targets on CD16 dynamics and hence on NK cytotoxicity. *Methods*. The chimeric Rituximab and the human Ofatumumab anti-CD20 Abs were used to opsonise the human CD20+ Raji cell line or primary B-CLL cells. Primary cultured NK cells derived from healthy donors or B-CLL patients were allow to interact with streptavidin-loaded opsonised targets for 30 min to 12 hours, magnetically isolated and tested for phenotypic, functional and biochemical analysis. *Result and Discussion*. We show that opsonised target-experienced NK cells undergo a progressive CD16 down-modulation. Unexpectedly, the sustained CD16 stimulation induces a major and persistent impairment of the spontaneous cytotoxic response against a panel of sensitive targets attributable to the cross-tolerance of several unrelated NK activation receptors including NKG2D, DNAM-1 NKp46 and 2B4. Notably, also in autologous setting, NK cell stimulation with Rituximab-opsonised B-CLL cells induces a marked CD16 down-modulation associated to a significant impairment of the cytolytic potential. NK hyporesponsive status correlates to CD16 down-modulation, but is largely independent from the exhaustion of cytolytic mediators or from the down-modulation of activation receptor expression. Moreover, the ability to form conjugates with sensitive targets and to polarise lytic granule at immune synapse is preserved; by contrast, degranulation is impaired. We are investigating the molecular mechanisms by which CD16 partecipates in spontaneous cytotoxicity, in particular the signalling network involved in the control of granule exocytosis. *Conclusions*. We propose a new mechanism of exhaustion of cytotoxic effectors that may impact on the immunocompetence of therapeutic-Ab treated patients.

C074

IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF ANTIBODY IMMUNE RESPONSES ELICITED BY SELF ANTIGENS IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Chronic lymphocytic leukemia (CLL) is characterized by extreme clinical and biological variability. Phenomena of immune dysfunction and autoimmunity have been described in CLL but little is known about antibody (Ab) immune responses. This study aims at understanding the functional role of humoral responses in CLL and specifically at 1) identifying circulating Ab in CLL pts' sera; 2) characterizing tumor antigens (Ag) recognized by serum Ab and 3) evaluating whether the identified immune responses have a cytotoxic effect toward leukemic cells. Proteins from CLL cells were separated to obtain proteomic maps which were blotted with autologous sera to reveal Ab-based reactivity. To verify the CLL-specificity of recognition, 8 pts maps were also probed with 8 healthy donors (HD) sera. Ag spots were identified by Mass Spectrometry (MS). Ag surface expression on viable and apoptotic cells, and C4 deposit were evaluated by flow cytometry. Complement-dependent cytotoxicity (CDC) was performed incubating CLL cells alone or in presence of alemtuzumab and subsequently with CLL pts' sera for 1,Ääh at 37,Ää°C. Cell viability was evaluated by Annexin-V/Propidium Iodide assay. Sera obtained from 35 untreated CLL pts were individually screened for the presence of IgG-based reactivity against autologous proteins extracted from leukemic cells. As a control, sera from 8 HD were probed on as many CLL maps. Overall, 29 out of 35 sera were immunoreactive and produced 145 Ag spots. By contrast, HD sera were significantly less reactive with 3 spots (p=.007). Pts with progressive CLL showed a significantly higher number of spots compared to pts with stable disease (p=.031). Sera from 54% of pts exhibited reactivity toward α-enolase (ENOA), whereas none of the HD sera was ENOA-reactive. Flow cytometry revealed that ENOA was not expressed on the surface of PB and BM CLL B cells at baseline conditions. We observed that the ENOA surface expression was significantly increased on apoptotic CD19+ CLL cells compared to baseline values (p<.01), but not on the CD19+ viable fraction, after 4 days of colture. ENOA Ab-reactivity significantly correlated with higher number of leucocytes, higher lymphocytosis and lower platelet counts (always p<0.01). The presence of specific anti ENOA, G3P, GDIR2 Ab was significantly correlated with the status of progressive disease (p=.02). We therefore performed a CDC assay to evaluate whether the humoral responses identified were capable of killing CLL cells. Results demonstrated that there was no C4 deposit on CLL cells surface and that CLL sera had no cytotoxic effect toward tumor cells. In this study we identified tumor Ag capable of eliciting humoral responses in CLL. Ab identified in most pts' sera were not able to induce CDC toward CLL cells and the Ab-based reactivity correlated with parameters of disease progression. Our results suggest that the immunoreactivity against self-Ag may be the expression of a dysfunctional immune system in CLL.

C075

HUMAN MONOCYTE-DERIVED DENDRITIC CELLS EXPOSED TO HYPERTHERMIA SHOW A DISTINCT GENE EXPRESSION PROFILE AND SELECTIVE UPREGULATION OF IGFBP-6

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Introduction. Dendritic cells (DCs) have been used for immunotherapy of cancer but their immunostimulatory functions are still incompletely understood. Fever plays a role in activating innate immunity while its relevance in activating adaptive immunity is less clear. Even brief exposure to elevated temperatures significantly impact on the immunostimulatory capacity of DCs, but the molecular mechanisms underlying this effect and their consequences on immune response remain unclear. *Methods.* We analyzed the gene expression profiles of normal human monocyte-derived DCs in paired samples from nine healthy adults subjected either to fever-like thermal conditions (39°C) or to normal temperature (37°C) for just 180 minutes. For each sample, total RNA was isolated and cDNA samples were amplified using the Platinum SYBR Green in an iCycler iQ Real Time Detection System. Secreted IGFBP-6 was measured using the Luminex xMAP technology. In order to evaluate chemotactic activity of IGFBP-6, monocytes, T and B lymphocytes were added in the upper compartment Transwells with 3 µm-pore filters, with different concentrations of IGFBP-6 (0.01 µg/ml, 0.1 µg/ml, 1 µg/ml) corresponding respectively to 0.4, 4, and 40 nM. After 150 min, cells that crossed were recovered in the lower compartment and quantified by flow cytometry, and data were expressed as percentage of migration of the control (100%). *Results.* Short exposure of DCs to 39°C caused upregulation of 43 genes and downregulation of 24 genes. Functionally, the up/downregulated genes are involved in post-translational modification, protein folding, cell death and survival, and cellular movement. Since we explored global gene expression profiling in order to search for early response genes involved in or governing the effects of hyperthermia on the immune system, it was intriguing that a few upregulated genes encoded secreted proteins, namely MANF, PLAT and IGFBP-6, which are potentially capable of interacting with other immune cells through receptors. Notably, when compared to monocytes, DCs differentially upregulated transcription of the secreted protein IGFBP-6, not previously known to be specifically linked to hyperthermia. In order to test whether IGFBP-6 is actually secreted by DCs after upregulation upon temperature stress, IGFBP-6 levels were

measured in supernatants from monocyte-derived DCs exposed for three hours at 37°C *versus* 39°C. Indeed, DCs exposed to 39°C secreted IGFBP-6 which was found to induce chemotaxis of monocytes and T lymphocytes, but not of B lymphocytes. *Conclusions.* Temperature regulates complex biological DCs' functions that most likely contribute to their ability to induce an efficient adaptive immune response. Our data indicate that IGFBP-6 may have relevance in cellular immunity, promoting the extravasation of monocytes and different lymphocyte subpopulations during the immune response.

C076

CHARACTERIZATION OF T-CELL SUBSETS IN ACUTE MYELOID LEUKEMIA PATIENTS AFTER INDUCTION CHEMOTHERAPY

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Introduction. AML is a very heterogeneous disease. Although its prognosis may be extremely different according to specific clinical and biological factors, survival of AML patients is about 50%. It is well-known that a good fraction of AML patients respond to first-line chemotherapy. However, chemotherapy is not often capable to eradicate the disease and leads to not curable relapse. Recent data support the role of some chemotherapeutic agents, *i.e.* anthracyclines, in activating the anti-tumor immune response in leukemias. This immunological process, known as cancer immunogenic cell death, synergizes with the pharmacological effect of antitumor drugs and may critically influence the clinical outcome of cancer patients. *Methods.* The peripheral blood and bone marrow from 16 AML patients undergoing anthracycline-based induction chemotherapy were analyzed. Before and at different time points after chemotherapy (7, 14, 21 and 28 days), the frequency of IFN-gamma producing CD4 and CD8 positive T cells, circulating T regulatory cells (Tregs) and leukemia-associated antigen-specific T-cells were stained. CD3+ T cells, isolated before and at different time points after chemotherapy, were cultured with pre-chemotherapy isolated leukemia blasts (and with autologous B cells as control samples) and tested for the production of IFN-gamma by flow cytometry. FOXP3+ Tregs were measured and correlated with that of leukemia-specific T cells. In HLA-A*0201+ patients effector T cells and Tregs with specificity for the Wilms' tumor antigen-1 (WT1) were evaluated using a sensitive dextramer-based staining. *Results.* 9/16 adult AML patients, undergoing doxorubicin-based induction chemotherapy, have an increase in leukemia-specific IFN-gamma producing, mainly CD8+, T-cells. The major increase of IFN-gamma production was observed at day 14 and 21 after chemotherapy. Interestingly, a significant fraction of these T cells were specific for the leukemia-associated antigen WT1, as stained in HLA-A*0201+ AML patients. Similarly, an increase of the number of FOXP3+CD4+CD25+ Tregs after chemotherapy was observed with a peak at day 14. Again, in HLA-A*0201+ AML patients, Tregs collected after chemotherapy showed an antigen-specificity for WT1. *Conclusions.* Here we evaluated the frequency of leukemia-specific Tregs and T effector cells in patients undergoing chemotherapy regimen containing anthracyclines. We observed an increase in leukemia-specific IFN-gamma-producing effector T cells and Tregs numbers after anthracycline-based chemotherapy. This finding may suggest that in AML, similarly to solid tumors, the anthracycline-based chemotherapy is highly efficient in eliciting anti-tumor immunity through the mechanism of immunogenic cell death. Further experiments addressing the mechanism(s) underlying such process are currently on-going.

C077

EPITHELIAL-TO-MESENCHYMAL TRANSITION (EMT) ELICITS MESENCHYMAL STROMAL CELL-LIKE IMMUNE MODULATORY PROPERTIES IN CANCER CELLS

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Introduction. Epithelial-to-Mesenchymal Transition (EMT) plays a central role in cancer progression and metastatic dissemination and may be induced by local inflammation. We asked whether the acquisition of mesenchymal phenotype by neoplastic epithelial cells is associated with the onset of mesenchymal stromal cell-like immune regulatory properties that may enhance tumor immune escape. *Methods.* Cell lines of lung adenocarcinoma (A549), breast cancer (MCF7) and hepatocellular carcinoma (HepG2) were co-cultured with T, B and NK cells before and after EMT induction by either the supernatant of mixed-lymphocyte reactions (MLR) or inflammatory cytokines (TGF-beta, INF-gamma and TNF-alpha). At the end of the co-culture the proliferation, the viability of the immune cells and the presence of regulatory population were evaluated respectively by CFDA-SE dilution, Annexin V-PI and a specific panel of antibody (CD4, CD25, FoxP3 or CD24,CD38). *Results.* Proinflammatory stimulation by MLR or by citokines induce EMT in the selected cancer cell lines. The cancer cells after EMT acquire multiple immune regulatory effects resulting, depending on the cancer cell line, in NK and T cell apoptosis, inhibition of lymphocyte proliferation and stimulation of regulatory T and B cells. Indoleamine 2,3-dioxygenase (IDO) resulted upregulated and involved in the immunomodulatory properties toward T cells, acquired by the cancer cell lines after EMT, as shown by the use of specific inhibitors. *Conclusions.* EMT induced by inflammatory stimuli confers to cancer cells some mesenchymal stromal cell-like immune modulatory properties, which could be a cue for cancer progression and metastatic dissemination by favoring immune escape.

C078

MECHANISMS OF Vγ9Vδ2 T-CELL ACTIVATION BY SOLUBLE METABOLITES GENERATED BY THE MEVALONATE PATHWAY

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Introduction. Phosphorylated metabolites of mevalonate (Mev) pathway such as isopentenyl-pyrophosphate (IPP) induce the proliferation of Vγ9Vδ2 T-cells. IPP generation can be lowered by statins or increased by aminobisphosphonates (NBPs) such as zoledronic acid (ZA) which target the Mev pathway. We have recently shown that dendritic cells treated with ZA (DCZA+) produce and release high amounts of soluble IPP and are very potent Vγ9Vδ2 T-cell activators. However, we have also shown that some tumor cell lines generate high amounts of IPP which is not released and do not activate Vγ9Vδ2 T-cells. The aims of this study were: 1) to investigate the ability of a variety of tumoral and non-tumoral cells to generate IPP and activate Vγ9Vδ2 T-cells; 2) to decipher some of the mechanisms involved in IPP release, the working hypothesis being that transporters involved in the efflux of cholesterol could also be involved in IPP release. *Methods*. We have tested a variety of tumoral and non-tumoral cells for their ability to activate Vγ9Vδ2 T-cells and correlated this ability with the release of extracellular IPP. Samples from healthy donors included peripheral blood (PB) monocytes, monocyte-derived DCs, and bone marrow (BM) stromal cells; samples from cancer patients included myeloma cells, chronic lymphocytic leukemia (CLL) cells, and BM stromal cells from both multiple myeloma (MM) and CLL patients; tumor cell lines included the monocytic THP1 and histiocytic U937 cells under baseline conditions or after differentiation into DCs (DC THP1, DC U937), and the myeloma cell line SKMMI. *Results*. We found that IPP efflux was very different with extracellular IPP ranging from picomolar to micromolar concentrations. The activation of Vγ9Vδ2 T-cells was proportional to the simultaneous efflux of IPP and to the activity/expression of ABCA1, a membrane transporter involved in the delivery of intracellular cholesterol to apoA-I. Treatment of ABCA1+ cells with the ABCA1 inhibitor probucol did not change the rate of intracellular IPP synthesis, but decreased IPP efflux and the ability of ABCA1+ cells to activate Vγ9Vδ2 T-cells. siRNA ABCA1 experiments confirmed that in the absence of ABCA1 protein the expansion of Vγ9Vδ2 T-cells is strongly reduced. *Conclusions*. These preliminary results suggest that ABCA1 is the protein involved in the efflux of IPP and therefore plays a crucial role as mediator of the immune responses mediated by Vγ9Vδ2 T-cells.

C079

INHIBITION OF THE PI3K/Akt/mTOR PATHWAY AT DISTINCT LEVELS HAS DIFFERENTIAL EFFECTS ON MONOCYTE-DERIVED APC

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Introduction. APC are essential to induce immune responses, such as graft *versus* host disease (GVHD). The PI3K/Akt/mTOR pathway is crucial in survival, proliferation and cell cycle progression of APC. Nowadays, there are several drugs inhibiting different target of this pathway. In this study we have compared the effects of the blockade of the PI3K/Akt/mTOR at different levels (Akt *versus* mTOR). *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-like cells and DC, respectively. Akt was inhibited by perifosine at 5 microM while mTOR was inhibited by rapamycin at 10 ng/ml. Both reagents were added at the start of culture. After 6 days cells were harvested and checked for their (1), apoptosis (2) expression of costimulatory and DC-differentiation molecules, (3) production of TNF-α and IL-12. *Results.* The phosphorylation of Akt after 2 hours of culture was completely inhibited by perifosine but not by rapamycin. Perifosine increased the apoptosis of monocytes cultured with GM-CSF only or with IL-4 (51±13% with *vs* 11±4% w/o perifosine and 33±14% with *vs* 19±9% w/o respectively, p<0,005). This effect was observed already on day 2. Instead, rapamycin increased the apoptosis of monocytes only in the presence of IL-4 (65±13% with rapamycin *vs* 42±21% w/o rapamycin after 6 days) while it had no detectable effect on the apoptosis of monocytes cultured with GM-CSF alone. Perifosine did not alter the phenotype of APC, while rapamycin decreased the expression of CD86 in a IL-4-dependent fashion. Finally, perifosine inhibited completely the production of both TNF-a and IL-12 by both macrophage-like cells and DC (by 90% and 70% for TNF-a p=0.006, respectively; and 90% and 30%, for IL-12, p=0.0028). (Instead, rapamycin inhibited the production of both TNF-a and IL-12 by IL-4 cultured APC but not by macrophage-like cells (by 89±7.7% *vs* 37±28% for TNF-a p=0.006; and by 85±30% *vs* 0%, for IL-12, p=0.0028, at day 6). *Conclusions.* These results suggest that Akt inhibition by perifosine alters the survival, the phenotype and the function of monocyte-derived APC independently of the presence or absence of specific growth factors in the culture medium, as opposed to mTOR inhbition by rapamycin, that that requires IL-4. Perifosine might be therefore more effective in APC inhibiton in the clinical setting to prevent GVHD.

C080

THE BONE MARROW REPRESENTS A SITE OF ENRICHEMENT OF PROTECTIVE T-LYMPHOCYTES AGAINST FILAMENTOUS FUNGI

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Introduction. Bone marrow (BM) has already been described as an enrichment site for several antigen-specific T cells, such as anti-viral and anti-tumor specific T cells, but the presence of specific T cells against filamentous fungi has never been investigated there. We have previously demonstrated mould-specific T cells in the peripheral blood (PB) of patients with either Invasive Aspergillosis (IA) or Mucormycosis (Potenza *et al.* Blood 2011 and PlosOne 2013). In PB, such T cells could be detected only during the course and tended to become undetectable after the resolution of the infection, therefore we have hypotized that BM could be an enrichment site for mould-specific T-lymphocytes. *Methods.* We used the Cytokine Secretion Assay (CSA) to evaluate and characterize the presence of mould-specific T lymphocytes secreting IFNg, TNFa, IL10, IL4, and IL17. Aspergillus fumigatus and Rhizopus oryzae germinated and heat inactivated conidia have been used as antigens. We tested paired samples of BM and PB from 7 patients with Invasive Fungal Infection (IFI) at different time points from the diagnosis of the infection (range 3-33 months). *Results.* CSA analysis revealed the presence of mould-specific T cells more frequently in BM than in PB. Mean frequencies (± standard deviation) in BM and PB resulted respectively: IFNg-producing T cells (3.24±6.10 *vs* 0.67±1.24), TNFa-producing T cells (0.43±0.59 *vs* 0.37±0.64), IL10-producing T cells (0.22±0.33 *vs* 0.00), IL17-producing T cells (0.16±0.18 *vs* 0.15±0.23) and IL4-producing T cells (0.59±0.63 *vs* 1.10±1.35). Mould-specific memory repertoire consisted of both CD4+ and CD8+ T lymphocytes with either Central Memory or Effector Memory phenotype. Of note, using a Wilcoxon signed rank test for paired samples, the observed differences in IFNg-producing T cells between BM and PB demonstrated statistically significant (P<0.05), and the contribution in the IFNg production of BM CD8+ T cells were significantly higher (P<0.05) than CD4+ T cells (mean±std CD8+ 2.64±5.73 *vs* CD4+ 0.59±0.45). Finally the frequencies of bone marrow Central Memory CD8+ T cells (mean 1.79±4.57) resulted higher than those of Effector Memory CD8 T cells frequencies (mean 0.87±1.18) but not in a statistical manner (Figure 1). *Conclusions.* This is the first report describing that the frequencies of protective mould-specific T lymphocytes producing IFNg are significantly higher in BM than in PB of patients with IFI. In particular these protective T cells are mainly represented by CD8+ T lymphocytes with Central Memory phenotype. The disappearance of such protective response due to mieloablative therapy could explain why bone marrow transplant and acute leukemia patients are particularly at risk for IFI. Such bone marrow specific T cells may represent the source for the expansion of Aspergillus-specific T cells to plan adoptive therapeutic strategies in patients with IA and candidate to bone marrow transplantation.

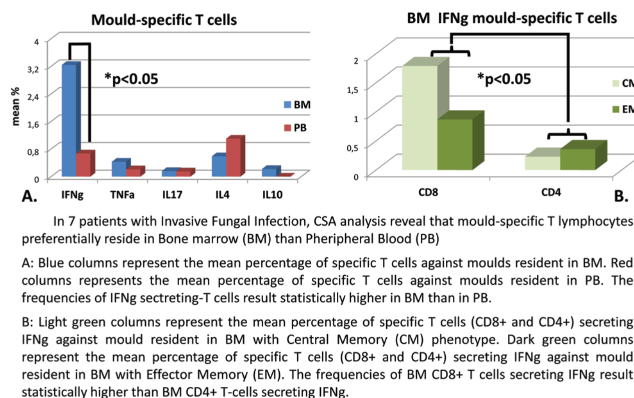

Figure 1.

Cytogenetic and Molecular Alterations

C081

COMPLEX CHROMOSOMAL REARRANGEMENTS LEADING TO EVI1 OVEREXPRESSION ARE RECURRENT MECHANISMS IN PATIENTS WITH VARIOUS 3Q ABNORMALITIES

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Introduction. Chromosomal rearrangements involving 3q26 region are recurrent findings in myeloid malignancies and lead to up-regulation of EVI1 gene that has been associated with a very poor prognosis. EVI1 is also overexpressed in subgroups of AML without 3q26 cytogenetic abnormalities, such as AML with -7/7q- abnormalities, which are also the most frequent additional abnormalities to EVI1 rearrangements. The aim of this study was to investigate EVI1 involvement in cases with myeloid malignancies and 3q or -7/7q- abnormalities. *Methods.* Patients with 3q or -7/7q- abnormalities by conventional cytogenetics (CC) were analysed by FISH with EVI1 breakapart probes and by relative RQ-PCR. *Results.* We analysed 107 AML, 32 MDS and 9 MPD cases with 3q (n=88) or -7/7q- (n=60) abnormalities. Among cases with 3q abnormalities, 26 showed inv(3)/t(3;3), 16 balanced 3q26 translocations, 11 balanced 3q21 translocations and 35 various 3q abnormalities involving different loci from 3q26. EVI1 rearrangements were detected in 36/42 (85.7%) cases with 3q26 abnormalities, whereas 6/42 (14.3%) had 3q26 abnormalities without EVI1 involvement. Unexpectedly, 4/11 (36.4%) cases with balanced t(3q21) displayed EVI1 rearrangements. In these latter cases, metaphase FISH analyses revealed that EVI1 rearrangements were the consequence of complex mechanisms involving multiple breakpoints on 3q arm that masked 3q26 involvement. Multiple breakpoints were also identified in 2 cases with t(3;8)(q26;q24) suggesting that this mechanism can occur in apparent classical 3q26 translocation, too. EVI1 rearrangement was also detected in 3/35 (8.6%) cases with various 3q abnormalities: metaphase FISH revealed a t(3;6)(q26;q25), t(1;3;13)(p34;q26;q14) and a t(2;3)(p21;q26) that were not observed by CC because of suboptimal quality of metaphases. All cases with EVI1 rearrangements showed overexpression by relative RQ-PCR. Among cases with 7/7q- abnormalities, only one case demonstrated EVI1 amplification by FISH. A further analysis of the karyotype allowed the identification of a sub-clone with 3q abnormality harbouring EVI1 gene amplification not previously identified. Other 8 cases showed elevated EVI1 expression without EVI1 rearrangements. Patients' clinical data are shown in Table 1.

Table 1.

		EVI+ n=44
Sex, (%)	Male	27 (61,4)
	Female	17 (38,6)
Age, years	Median (range)	51 (16-83)
WBC x10⁹/L	Median (range)	12,5 (1,3-167)
Platelets x10⁹/L	Median,(range)	120 (12-1140)
Hb (g/dl)		9,2 (4,7-12,4)
'de novo' AML		23 (52,3%)
secAML		11 (25%)
'de novo' MDS		4 (9,1%)
secMDS		4 (9,1%)
MPD		1 (2,3%)
Cytogenetics, n(%)	(-7/7q-)	26 (59,1%)
	(-5/5q-)	7 (15,9%)
	complex karyotype	10 (22,7%)
	isolated abnormality	14 (31,8%)
CCyR (%)		20,8
Survival	Median (months)	10,6
Survival	years	
	1	33%
	3	5%
	5	5%

Conclusions. Although 3q26 abnormalities are strictly associated with EVI1 rearrangements, FISH and RQ-PCR identified cases with 3q26 abnormalities involving EVI1 and cases with EVI1 rearrangements without 3q26 involvement by CC. These events could be the consequence of a low quality of metaphases, but they were often the result of complex chromosomal rearrangements. Because of poor prognosis of EVI1 overexpression, a screening for EVI1 rearrangements should be performed in myeloid malignancies with chromosome 3q abnormalities. Furthermore, FISH should be performed in cases with 7q abnormalities when the quality of metaphases is suboptimal. Supported by University of Bologna RFO, BolognAIL and Coop Reno.

C082

NOVEL CHIMERIC TRANSCRIPTS INVOLVING PAX5 IN B-CELL PRECURSOR ALL

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Introduction. PAX5, located on 9p13, belongs to the PAX gene family and encodes for a transcription factor essential for B lymphoid cell commitment. It functions both as a transcriptional activator and repressor of different target genes involved in lineages development. PAX5 has been recently reported to be target of aberrancies (including point mutation, deletions, and gene fusions), in approximately 30% of pediatric patients affected by BCP-ALL, the most frequent leukemia subset in children. Translocations are estimated to occur at an incidence of 2-3%, with a variety of partner genes, encoding for transcription factors (TEL, PML, FOXP1), kinases (JAK2), structural proteins (ELN, POM121) or molecules of unknown function (C20orf112, AUTS2). *Methods*. FISH analysis was performed on bone marrow cells of all cases to dissect the genomic breakpoints and the structure of the rearrangements. The fusion genes were cloned by 5' and/or 3' RACE PCR, confirmed by sequencing and verified by RT-PCR with specific primers on the source material. PAX5-translocated cases were further characterized by genome-wide CytoScan HD SNParray (Affymetrix). *Results*. We performed a FISH-based study on an Italian cohort of BCP-ALL patients having 9p13 chromosomal rearrangement (as a hallmark of PAX5 rearrangement), and we identified novel PAX5 partner genes. Three novel partner genes of PAX5 were identified by FISH. SOX5 was found as a PAX5 partner in a pediatric patient harboring a dic(9;12)(p13;p13) chromosome. A further patient, showing a t(9;12)(p13;q34) translocation, revealed PAX5 as fused to a novel transcript isoform of CHFR, a gene widely expressed in a library of normal tissues. A third partner was identified in an adult B-ALL case, which showed a deletion within the short arm of chromosome 9, leading to the fusion of PAX5 to MLLT3. A fourth PAX-rearranged case, involving POM121C (different from the already described POM121) as fused to PAX5 in a t(7;9)(q11;p13) translocation, was identified by a RNAseq approach on BCP-ALL cases without known prognostic features. Moreover, two pediatric patients were harboring a t(7;9)(q11.2;p13.2) with a PAX5/AUTS2 fusion transcript, thus confirming its recurrent alteration in pediatric B-ALL. Interestingly, Copy Number Variation analysis showed that a limited number of cooperative genetic lesions were present in addition to the translocation event, with a mean of 2.4 events for each patient. *Conclusions*. We therefore hypothesize that PAX5 rearrangements may have a a primary role, representing single genetic aberration events in a simple background, rather than being part of a complex scenario of cooperating genetic lesions involved in leukemogenesis. A common pathway for all PAX5 genomic lesions still need to be elucidated.

C083

TRANSCRIPTOMIC ANALYSIS (RNA-SEQ) TO DISSECT THE BIOLOGY OF DIFFERENT THERAPY RESPONSE IN CHILDHOOD ALL PATIENTS

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Introduction. Acute Lymphoblastic Leukemia (ALL) is the most frequent type of childhood leukemia. It is a multi-step process, characterized by the expansion of a pre-leukemic clone, accumulating cooperative genetic events required for the full transformation and clinical manifestation. Recently, the technological advances in genome-wide profiling techniques have allowed a better understanding of its molecular basis and heterogeneity. However, incidence and cure rates greatly differ among children, reflecting diverse responses to drug treatment and distinguishing risk groups. Molecular investigations addressing such individual differences will help to better understand leukemia biology and to improve risk prediction. We applied a whole-transcriptome sequencing approach (RNA-seq) to characterize low- (LR) *versus* high-risk (HR) patients to identify new genetic explanations for their different early response to therapy. Moreover, we focused on the identification of novel pre-leukemic and leukemogenic events. *Methods*. Total RNA was extracted from primary leukemic blast samples of 10 pediatric ALL patients (4LR and 6 HR, according to minimal residual disease monitoring), included in the Italian AIEOP-BFM ALL2000 protocol. Genome-wide DNA profiling was performed by Affymetrix Cyto2.7M Arrays, RNA-seq was carried out by an Illumina GAIIx platform, and validations were performed by RT-PCR and FISH. *Results*. We analyzed the transcriptome of 10 childhood ALL cases, not carrying any other clinical or genetic risk factor. For each case, we identified fusion transcripts, alternative splicing and SNPs. Priority was given to putative fusion transcripts, which could originate from intra- or inter-chromosomal rearrangements. We identified 127 fusion events. Strikingly 123 out of 127 events were identified as intra-chromosomal fusions, 117 of which were involving two contiguous genes or with overlapping loci (the so-called “con-joined genes”).Among the intra-chromosomal events, the NUP214-ABL1 fusion, previously found in T-ALL and responsive to kinase inhibitors, was identified and validated in one HR patient. Finally, among the 4 inter-chromosomal fusions, the novel PAX5-POM121C fusion was identified and validated in one LR patient. Potential interest for leukemia was explored by testing these events also in other RNA-seq datasets from different tumors and normal blood samples. *Conclusions*. RNA-seq represents one of the most comprehensive approaches to identify genetic alterations carried by leukemia clones. Our analyses detected novel fusion genes, originated either by inter- or intra-chromosomal rearrangements, as well as a considerable number of con-joined genes. Further evaluations will address SNPs, mutations, gene expression changes and splice variants that could be related to a different risk of relapse, and the feasibility of the screening of the candidates on a larger population of consecutive cases.

C084

TARGETED NEXT GENERATION SEQUENCING ANALYSIS REVEALED THE PRESENCE OF LESIONS IN B-CELL GENES INVOLVED IN LEUKEMIA, IN THE “PHILADELPHIA-LIKE” GROUP IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

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Introduction. Up to 20% of pediatric patients affected by B-cell precursor ALL (BCP-ALL) experience relapse. A major effort is dedicated to identifying poor prognostic subgroups in the subset of patients with intermediate-risk, where most relapses occur. Recently, a novel ALL subtype with high incidence of relapses (‚ÄòPh-like’) has been identified, with a gene-expression profile (GEP) similar to Philadelphia chromosome-positive ALL, however in the absence of the Ph-chromosome. The principal aims of this project are to identify BCP-ALL Ph-like cases in the Italian ALL AIEOP cohort and to define major genetic abnormalities and discover new aberrancies. *Methods.* Gene Expression and CytoScan HD SNParray (Affymetrix); MLPA probemix P335-B1 ALL-IKZF1 (MRC-Holland); Miseq platform (Illumina) using Nextera Rapid Capture Custom panel. *Results.* Among 400 Italian childhood BCP-ALL cases enrolled in AIEOP-BFM protocols, 143 patients, negative for all the known common alterations, were identified as B-others. 43/143 B-others cases had a GEP signature similar to the BCR/ABL1 patients, and named Ph-like. The 5y event-free survival (EFS) of Ph-like patients was 54.8% *vs* 83.1% in the remaining B-others patients ($p<0.001$), due to an increased incidence of relapse. Several approaches have been developed to analyze genomic lesions. We developed a novel NGS panel to discover genetic lesions (deletions, point mutations and fusion genes) involving 17 genes, focusing specifically on genes fundamental both for B-cell development and leukemogenesis. We implemented a custom panel on Miseq, which allowed the rapid detection of alterations, performing a deep analysis on a region of about 1.2MB with a coverage of 99% and using 5361 probes. Moreover, Copy number Variation analysis and MLPA detected alterations on 14 target genes, some of them overlapping with NGS results. In particular, we detected deletions affecting PAX5 and CDKN2A-2B genes were found in 27/42 patients (64%). Moreover, 10/42 (26%) had IKZF1 deletions and the same frequency was found for the P2RY8-CRLF2 deletion. In addition, we found JAK2 gene lesions in 9 patients and VpreB focal deletion in 9 other patients (in 3/9 both lesions were present). In few cases BTLA/CD200 (N=5), BTG1 (N=6) and ABL1 genes (N=3) were affected. Furthermore, 2 samples presented a focal deletion on chromosome 5, fusing EBF1 to PDGFRB. The genetic lesions were not mutually exclusive, whilst the majority of patients had from 3 to 6 aberrancies. The majority of the alterations represented deletion events rather than amplification. *Conclusions.* We identified a Ph-like subgroup in the Italian cohort of children with BCP-ALL, associated to a poor outcome. The genetic characterization of this subgroup showed the frequent involvement of B-cell genes, with each patient having multiple CNVs. Dissecting the genomic scenario will allow to elucidate the molecular and biological pathways to design new strategies for targeted drugs.

C085

HAPLO-INSUFFICIENCY OF NPM1 GENE AND CHROMOSOMAL INSTABILITY AFTER CHEMICAL AND PHYSICAL INSULTS

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Introduction. NPM1 gene is a nucleolar phosphoprotein with the function of molecular chaperone shuttling between the nucleolus and the cytoplasm. Inappropriate expression of NPM1 gene as well as its involvement in genomic rearrangements were found in human solid and hematological tumors. One of us (C.M.) previously discovered exon 12 NPM1 mutations in around 60% of adult AML with normal karyotype and showed that in 45% of AML with complex karyotypes including -5/5q-, NPM1 gene at 5q35 is lost and haplo-insufficient with 47% decrease of expression (NEJM 352(7):740,2005; PLosOne 5(9):e12855,2010). Notably, in AML with complex karyotypes the category of therapy-induced leukemias is consistently represented and -5/5q- are concentrated in AML arising after alkylating agents (Chem Biol Interact 184(1-2):50,2010). In this work we aimed: 1) to generate NPM1 haplo-insufficiency in a human cell line; and 2) to investigate the effects of clastogens. *Methods*. We used the transformed human diploid fibroblast cell line (MRC-5). Silencing was obtained by specific interfering RNA (B23 lentiviral particle, Santa Cruz) and confirmed by qRT-PCR using TaqMan assay probes (Applied Biosystems) Hs02339479_g1 for NPM1 and Hs00245445_m1 for the reference control ABL1. Both MRC-5 and siNPM1-MRC5 cell lines were treated with different DNA damaging agents, such as camptothecin (CPT), ultraviolet light (UV) and ionizing radiation (IR). Chromosomal damage was evaluated by chromosomal aberrations (CA) and sister chromatid exchanges (SCE). Primary DNA damage and repair were investigated by comet assay. Cell

death was estimated using viable and non viable cell stainings. *Results*. qRT-PCR revealed a 30% decrease in NPM1 expression in the silenced cell line compared to the wild-type MRC5. Cytogenetic analysis showed a strong increase in chromosomal damage in the siNPM1-MRC5, as compared to the MRC5 cell line. The silenced cell line was sensitive to all chemical and physical agents, namely CPT, UV and X-rays. X-rays exposure never induced apoptosis nor necrosis. The higher sensitivity of the siNPM1-MRC5 was not related to cell cycle delay or cell death. According to the comet assay primary DNA damage and repair kinetics were not affected. *Conclusions*. Our data validated the siNPM1-MRC5 cell line as an *in vitro* model for the *in vivo* NPM1 haplo-insufficiency of AML with 5q- in complex karyotype. The silenced cell line wass significantly sensitive to all the DNA damaging agents we used in this study. These results prove a dosage effect of NPM1 to maintain chromosomal stability and strongly support a critical role of NPM1 deletion/haplo-insufficiency in the origin and/or perpetuation of gross chromosomal aberrations in human AML.

C086

TWO NOVEL METHODS FOR RAPID DETECTION AND FOR QUANTIFICATION OF DNMT3A R882H MUTATION IN ACUTE MYELOID LEUKEMIA

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DNMT3A mutations represent one of the most frequent gene alterations detectable in Acute Myeloid Leukemia (AML) with normal karyotype (NK). While various recurrent somatic mutations of this gene have been described, the most common mutation is located at aminoacid R882 in the methyltransferase domain of the gene. DNMT3A mutations have been reported to be stable during disease progression and are associated with unfavorable outcome in AML patients with NK. Due to their prognostic significance and high stability during disease evolution, DNMT3A mutations might represent highly informative biomarkers for minimal residual disease monitoring. We describe here two novel assays for the detection and quantification of DNMT3A R882H mutation in AML patients. Bone marrow samples were collected at diagnosis from 134 AML patients observed and treated with standard chemotherapy at the Department of Biomedicine and Prevention of the University Tor Vergata of Rome between 2012 and 2014. For the diagnostic screening of DNMT3A R882H we used a strategy based on TauI enzyme digestion followed by capillary electrophoresis. TauI recognizes and cuts the wild type (wt) R882 sequence while the presence of R882H mutation abrogates the TauI cleavage site (Figure 1 A). To evaluate the DNMT3A R882H kinetics in AML mutated patients, we developed a method based on peptide nucleic acid (PNA) Real Time PCR (RQ-PCR) in which the amplification of the wt allele of DNMT3A is inhibited and only the mutant allele (R882H) is amplified (Figure 1 B). The RQ-PCR assay for DNMT3A R882H quantification was designed using two different probes: a mutant fluorescent probe with the identical sequence of the DNA region containing the mutation and a PNA probe to block the wt allele amplification With our PCR assay followed by TauI restriction enzyme digestion we identified 24 DNMT3A R882H mutated patients out of 134 AML screened samples (17%). All DNMT3A wt patients revealed only one fragment of 93 bp as the result of enzymatic digestion, while DNMT3A R882H mutated patients showed two different fragments of 93 bp and 145 bp, in keeping with the heterozygous state of DNMT3A R882H mutation (Figure 1 C). In addition, through our PNA-based RQ-PCR assay, we analyzed the kinetics of DNMT3A R882H after induction and after consolidation therapy in mutated patients. At diagnosis, DNMT3A R882H median transcript level was 1101.5/104 ABL1 copies, after induction and consolidation therapy the median transcript level decreased to 127/104 ABL1 copies and 76.5/104 ABL1 copies, respectively. In conclusion, our novel molecular tests for rapid diagnosis and monitoring of DNMT3A gene mutations may be helpful to improve diagnostic work-up and to and to evaluate disease evolution in AML with DNMT3A R882H. Further prospective longitudinal studies in large cohorts of AML patients are needed to investigate the clinical impact of DNMT3A R882H mutation on the outcome of the disease, in term of response to therapy and prediction of disease relapse.

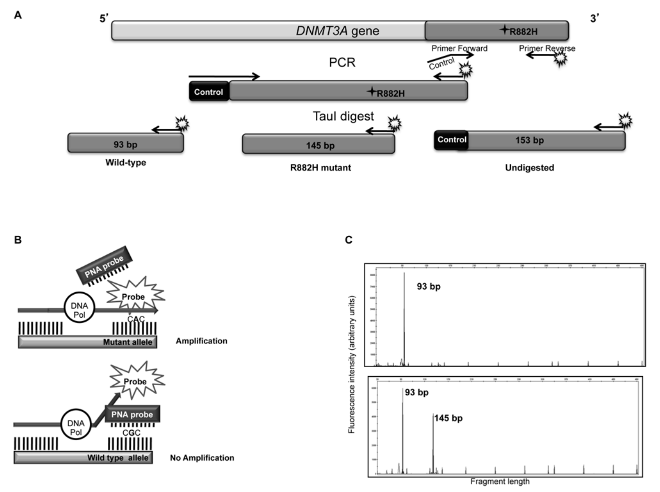

Figure 1. Diagram of TauI restriction enzyme assay to identify *DNMT3A* R882H mutation (Panel A). A schematic representation of a Peptide Nucleic Acid Real Time PCR technology for monitoring of *DNMT3A* R882H mutation (Panel B). Electropherogram on *DNMT3A* wt and mutated patient who show two peaks at 93 bp and 145 bp corresponding to the wt and mutated allele, respectively (Panel C).

C087

RARE IGH TRANSLOCATIONS IN NEWLY DIAGNOSED MULTIPLE MYELOMA (MM): CYTOGENETIC CHARACTERIZATION AND PROGNOSITC ROLE IN 595 PATIENTS

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Introduction. Approximately 50% of MM pts are characterized by translocations into the Immunoglobulin Heavy chain locus(IgH). Each translocation is associated with deregulation of a D group cyclin, either directly in t(11;14) and t(6;14) or indirectly in t(4;14) and in MAF translocations: t(14;16) and t(14;20). Some translocations are recurrent but rare events, occurring in less than 5% of the pts. Translocation t(6;14) is considered a standard prognostic feature, whereas t(14;16) and t(14;20) are associated with bad prognosis but, because of their low incidence, few data support their use in the risk stratification. The aim of our study was to assess the type and frequency of the rare IgH translocations and their association with clinical parameters in a large series of homogenously treated MM pts. *Methods.* A total of 595pts with newly diagnosed MM, enrolled in EMN02-HOVON95 clinical trial, were included in this study. Probes for FISH were chosen to detect t(4;14), t(6;14), t(11,14), t(14;16), t(14;20); deletion of 1p32, 13q14, 17p13; gain of 1q21; trisomy of chromosomes 5/9/15. *Results*. The frequency of the rare translocations detected by FISH was as follows: t(6;14) in 6/595 pts(1%), t(14;16) in 23/595(3.9%) and t(14;20) in 8/595(1.3%). Every pt carried the translocation associated with deletions flanking the breakpoints, either as unique pattern or with classical translocation. The t(6;14) was associated with del(13q)(4/6) and del(16)(q23)(3/6). All together, the high-risk translocations, t(14;16) and t(14;20), were detected in 5.2% of pts(31/595). The t(14;16) was related to the presence of del(13q)(82.6%;19/23) and gain(1q)(69.6%;16/23), while t(14;20) was associated with del(13q)(87.5%;7/8), del(16q)(4/8) and gain(1q)(4/8). Beyond del(13q), gain(1q) was the most frequent additional abnormalities(64.5%;20/31). None of the pts showed translocation as single abnormality. No significant differences in the main clinical features

between the group with t(14;16) or t(14;20) and the pts without these translocations emerged: age(55.5 *vs* 56.3); ISS stage 2-3(60% *vs* 57.7%); reactive protein C(5.9 *vs* 6.4); LDH(260.1 *vs* 281.6); bone lytic lesions(69.8% *vs* 77.6%). Time to progression(TTP) and progression-free survival(PFS) were shorter in pts with t(14;16) or t(14;20) (HR=3.7 and HR=3) in comparison to pts without the translocations. In multivariate analysis, the presence of t(14;16) or t(14;20) was an independent prognostic factor for reduced PFS(HR=3.3, p<0.01). With a median follow-up of 12 months, progressive disease was observed in 6 pts(26%;6/23) with t(14;16) and in 5 pts(62.5%;5/8) with t(14;20). *Conclusions*. t(14;16) and t(14;20) are rare in newly diagnosed MM pts, associated with additional high-risk (gain1q) abnormalities; on the contrary they are not apparently related to specific baseline clinical features. Pts carrying these abnormalities seem to display a poor prognosis. A longer follow-up is needed to point out their prognostic relevance.

C088

ANALYSIS OF STAT3 MUTATIONS IN PATIENTS AFFECTED BY LYMPHOPROLIFERATIVE DISORDERS OF LARGE GRANULAR LYMPHOCYTE

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Introduction. T large granular lymphocytes leukemia (T-LGLL) and NK-type chronic lymphoproliferative disorder (CLPD-NK) are rare diseases characterized by the abnormal expansion of large granular lymphocytes (LGL). The majority of patients are asymptomatic, although neutropenia or autoimmune rheumatic diseases can be reported in a significant percentage of cases. Actually, the pathogenesis of these disorders is still unknown. Several data indicate that LGL proliferation is maintained through the activation of many signaling pathways, among them JAK/STAT pathway has a relevant role in LGL survival and proliferation. Recently, both in T-LGLL and CLPD-NK, somatic STAT3 mutations have been reported in the SH2 domain, mostly represented by Y640F and D661Y. The aims of this study include: i) the analysis of STAT3 point mutations in T-LGLL and CLPD-NK patients DNA samples; ii) the evaluation of these mutations over time (mean follow-up 6 + 3 y); iii) the correlation between the presence of STAT3 mutations and clinical (in particular neutropenia) and biological (namely immunophenotypic) features of patients' LGL. *Methods*. One hundred one patients were included in the study: 60 affected by T-LGLL and 41 by CLPD-NK. Peripheral blood mononuclear cell (PBMC) immunophenotype was analyzed by flow cytometry for CD3, CD16, CD57, CD56, CD4, CD8, CD5, CD19, γδ, Vβ and Killer Immunoglobulin like Receptor (KIR) markers. For molecular analysis, LGLs were purified by microbeads or FACSAria cell sorting and DNA samples were sequenced by Sanger method. In addition, DNA samples were analyzed by Amplification Refractory Mutations System (ARMS-PCR) for Y640F and D661Y STAT3 mutations. *Results*. By the combination of Sanger sequencing and ARMS-PCR, 14/101 patients were found mutated in STAT3: 11 with T-LGLL (18% of all T-LGLL patients analyzed), and 3 with CLPD-NK (7% of all CLPD-NK patients analyzed). By flow cytometry we defined a discrete immunophenotype of LGLs in mutated T-LGLL patients characterized by CD8+, CD57+, CD16+ expression, a restricted TCR BV repertoire or KIR expression and more than 55% of total lymphocytes represented by LGLs. In all mutated patients mutations have been detected also during the follow-up. Furthermore, we found that 9/11 (82%) of T-LGLL mutated patients were characterized by neutropenia (ANC<1,000/μl), in particular 5/11 (45%) presenting with severe neutropenia (ANC<500/μl). *Conclusions*. Although the clinical relevance of STAT3 mutations is not yet completely clarified in these disorders, our results indicate that patients carrying STAT3 mutations might represent a subset with clinical and biological features suggestive for a more symptomatic disease.

Chronic Myeloproliferative Disorders

C089

SYSTEMATIC CORONARY RISK EVALUATION (SCORE) CHART IDENTIFY CHRONIC MYELOID LEUKEMIA PATIENTS AT RISK OF CARDIOVASCULAR DISEASES DURING NILOTINIB TREATMENT

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Nilotinib is currently approved for the treatment of chronic myeloid leukemia (CML) in chronic (CP) and accelerated phase (AP) after failure of imatinib and in newly diagnosed patients. Atherosclerotic events were retrospectively reported in patients with baseline cardiovascular risk factors during nilotinib treatment. We estimated the risk of developing atherosclerotic events in patients treated with second or first line nilotinib, with a median follow-up of 48 months, by retrospectively applying the SCORE chart proposed by the European Society of Cardiology (ESC) and evaluating risk factors at baseline (diabetes, obesity, smoking and hypertension). Overall, we enrolled in the study 82 CP patients treated frontline (42 patients, at the dose of 300 mg BID) or after failure of other tyrosine kinase inhibitors (40 patients, treated with 400 mg BID). The SCORE chart is based on the stratification of sex (male *vs* female), age (from 40 to 65 years), smoker *vs* non-smoker, systolic pressure (from 120 to 180 mm Hg) and cholesterol (measured in mmol/l, from 150 to 300 mg/dl). For statistical purposes we considered patients subdivided in low, moderate, high and very high risk. There were 48 males and 34 females, median age 51 years (range 22-84). According to WHO classification, 42 patients were classified as normal weight (BMI < 25), 26 patients were overweight (BMI 26- <30) and 14 were obese (BMI > 30). Retrospective classification according to the SCORE chart revealed that 27 patients (33%) were in the low risk category, 30 patients (36%) in the moderate risk category and 24 patients (29%) in the high risk category. As regards risk factors, we revealed that 17 patients (20.7%) had a concomitant type II controlled diabetes (without organ damage), 23 patients (28%) were smokers, 29 patients (35%) were receiving concomitant drugs for hypertension, 15 patients (18%) had concomitant dyslipidaemia. Overall, the cumulative incidence of atherosclerotic events at 48 months was 8.5% (95% CI: 4.55-14.07): none of the low-risk patients according to the SCORE chart experienced atherosclerotic events compared to 10% in the moderate risk and 29% in the high risk category (p=0.002). Atherosclerotic-free survival was 100%, 89% and 69% in the low, moderate and high-risk population, respectively (p=0.001). SCORE chart evaluation at disease baseline could be a valid tool to identify patients at high risk of atherosclerotic events during nilotinib treatment.

C090

DASATINIB FIRST-LINE: MULTICENTRIC "REAL-LIFE" EFFICACY AND SAFETY DATA

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Dasatinib was approved for the treatment of chronic phase (CP) chronic myeloid leukemia (CML) patients in first line, based on the demonstration of efficacy and safety were reported in patients enrolled in clinical trials. We describe here a multicentric evaluation of dasatinib used as first line treatment, in order to confirm data reported by phase III DASISION trial. Eighty-seven patients received dasatinib as first line treatment from January 2012: there were 49 males and 38 females, median age 56 years (range 24-90). Five patients received previous chemotherapy and radiotherapy for other tumours. According to ECOG, 58 patients had score 0, 27 had score 1 and 3 had score 2. Median WBC count at diagnosis was 74 x 109/l (range 15.3-401). Prognostic scores stratification was as follows: according to Sokal risk, 26 patients were low risk, 41 intermediate and 20 high risk; according to Hasford score, 35 patients were low risk, 38 intermediate and 10 high; according to Eutos score, 75 patients were classified as low and 12 as high risk. Eight patients had abnormal cytogenetic aberration (ACA) in the Ph+ clone at baseline. Sixty patients received prior cytoreductive therapy, and median time from diagnosis to start of dasatinib being 18 days. Only 4 patients received unscheduled dose (1 patients 50 mg and 3 patients 80 mg QD), whereas 84 patients started with 100 mg QD. At 3 months, 55 patients (63%) achieved CCyR, while cytogenetic evaluation was not available for 23 patients (15 not evaluable, 8 patients not done). Median BCR-ABL/ABL ratio at 3 months (evaluable in 72 patients) was 0.35% IS: MMR was achieved in 18 patients and EMR (ratio < 10%) was not reached only in 7 patients. At the end of the third month, 8 patients reduced the dose to 80 mg (4 patients) or to 50 mg (4 patients). At 6 months, rate of CCyR was 93% and median BCR-ABL/ABL ratio was 0.01% IS. Overall, 66 patients were evaluable for molecular response: 43 patients (65%) had a ratio < 0.1% with 16 patients being in MR4 and 27 patients showing a response between MR3 and MR4. Eight patients continued to receive a dose less than 100 mg QD. At 12 months (29 evaluable patients), rate of CCyR was 81%; median BCR-ABL/ABL ratio was 0.002% IS. Nineteen patients (65%) had a ratio < 0.1% and of these, 12 patients achieved an MR4.5. At last assessment, 11 patients were treated with low dose and 5 patients changed treatment (4 patients switched to nilotinib and 1 patient to imatinib). Two patients experienced a lymphoid blast crisis and both underwent allogeneic bone marrow transplant. As regards safety, 16 patients (18%) experienced thrombocytopenia, which was of grade 3 in 9 patients (56%); 7 patients (8%) experienced neutropenia, which was of grade 3 in 3 patients. Fifteen patients experienced non-hematologic side effects: of these 4 (4.5%) were pleural effusions, managed with temporarily discontinuation followed by dose reduction in 3 patients. Present results confirm efficacy and safety data previously reported for patients enrolled in clinical trials.

C091

BONE MARROW (BM) MICROENVIROMENT FACTORS AS EARLY MARKERS OF RESPONSE IN PATIENTS WITH NEWLY DIAGNOSED CHRONIC PHASE CHRONIC MYELOGENOUS LEUKEMIA (CML-CP) TREATED WITH NILOTINIB

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Introduction. Treatment of patients with CML-CP with tyrosine kinase inhibitors (TKIs) can substantially improve the life expectancy of these patients. However, it is becoming evident that persistent leukemic stem cells, which in their quiescent state are insensitive to TKIs, can lead to a resurgence of CML. The Evaluating Nilotinib Efficacy and Safety in Trial as First-Line Treatment (ENEST1st) is a phase 3b is an open-label study of nilotinib 300 mg twice daily (BID) in adults with newly diagnosed BCR-ABL positive CML-CP. The aim of the ENEST1st sub-study N10 is to investigate BM microenvironment markers that regulate leukemic stem cells in the BM niches of nilotinib-treated patients. *Methods*. We enrolled 37 patients from 21 Italian ENEST1st centers, from whom written informed consent had been obtained, for participation in sub-study N10. Patients were monitored by Real Time RT-PCR (RT-QPCR) for the expression of the fusion BCR-ABL mRNA. Response was based on ELN recommendations (Baccarani M, *et al.* Blood 2013 122:872-884). In an interim analysis, molecular and cytogenetics response by 24 months was assessed. Mononuclear cells were collected from BM and PB samples at the screening visit (V0) and after 3 months of treatment (V4). Total RNA from BM and PB mononuclear cells was purified. RT-qPCR for the expression of 10 genes (ARF, KIT, CXCR4, FLT3, LIF, NANOg, PML, PRAME, SET and TIE), involved in the regulation of the stemness and survival signaling of hematopoietic stem cells was conducted. RT-qPCR results were normalized by the expression of GUS mRNA (Normalized mRNA copy Number: NCN). *Results*. Interim molecular analysis of MMR until the 24th month was available for 27 of the 37 patients, showing an optimal response in 20 patients, a warning response in 4 patients and a failure response in 3 patients. We observed a significant correlation in the expression of two genes involved in the regulation of stem cell pluripotency (NANOg) or cytokine signaling (SET) and patient's outcome. Indeed, NANOg and SET mRNA were significantly down-regulated in PB samples at diagnosis of patients with optimal response compared to patients with warning/failure response, (NANOg mRNA: 0.3±0.25 NCN by GUS mRNA *vs* 0.6±0.7 NCN by GUS mRNA, respectively; p=0.05; SET mRNA: 0.2±0.3 NCN by GUS mRNA *vs* 2.3±4.2 NCN, respectively; p=0.03). *Conclusions*. These data suggest that expression analysis of genes involved in cell pluripotency (NANOg) and/or cell signaling (SET) at baseline, may assist in the early prediction of molecular response in patients treated with nilotinib. Further studies are planned to evaluate the role of stroma-secreted cytokines, such as SDF1 and VEGF, in the regulation of TKI-responsiveness in CML patients, since factors that modulate hematopoiesis may also promote leukemogenesis, enhance blast survival and make them resistant to treatment within the BM microenvironment.

C092

PREDICTORS OF DEEP MOLECULAR RESPONSE IN CHRONIC MYELOID LEUKEMIA PATIENTS TREATED FRONTLINE WITH IMATINIB MESYLATE: AN ANALYSIS BY THE GIMEMA CML WP

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Introduction. Imatinib mesylate (IM) has been for many years the standard of care for chronic myeloid leukemia (CML) in early chronic phase (CP). Initial treatment with IM produce a high probability of long-term survival, but few patients are able to achieve a sustained deep molecular response (MR4.0 or better) that is the pre-requisite to discontinue the TKI treatment. The treatment-free remission (TFR) will become very soon most important goal of CML treatment. *Methods.* The aim of the present study is to investigate the predictors of MR4.0 in CML patients treated frontline with imatinib. We analyzed 559 patients enrolled within 3 multicentric prospective studies conducted by the GIMEMA CML WP (NCT00514488, NCT00510926, observational trial CML023). Definitions: major molecular response (MMR), BCR-ABLIS ratio <0.1%; deep molecular response (MR4.0), detectable disease ≤ 0.01% BCR-ABLIS or undetectable disease with

≥10,000 ABL transcripts; stable MR4.0: MR4.0 lasting > 24 months and > 3 evaluable samples. All the analysis were made according to the intention-to-treat principle. *Results.* Baseline demographics characteristics: median age: 52 years (extremes 18-84 years); male/female sex: 60%/40%; high Sokal, high Euro and high EUTOS scores: 22%, 7% and 7%, respectively; clonal chromosomal abnormalities (CCA) in Ph+ cells: 4% (not evaluable in 32% of patients for insufficient number of metaphases); e13a2 BCR-ABL transcript: 36%. Median follow-up: 76 (7-99) months. The cumulative incidence of MMR and MR4 was 86% (95% CI: 83-89%) and 66% (95% CI: 62-70%), respectively. The median time to MMR and MR4 was 8 and 42 months, respectively. The rate of MR4.0 at 24, 36, 48 and 60 months was 18%, 27%, 30% and 33%, respectively. At the last contact, 25% of patients had a stable MR4.0. In a multivariate Cox analysis including baseline variables, male sex, larger spleen size and e13a2 transcript type resulted independent poor prognostic factors on the probability to achieve a MR4.0. Female sex was the only baseline variable able to predict a stable MR4.0. The reduction of BCR-ABL1 transcript levels and the time to molecular response are influenced by baseline variables, so the impact of the dynamics of molecular response on the probability of MR4.0 was analyzed separately. The time to MMR strongly predict both the achievement and the stability on MR4.0. *Conclusions.* As suggested by a previous report (Branford *et al.* Blood 2013), in a large nationwide multicentric experience, the female sex was confirmed as a predictor of the achievement of a stable deep molecular response. Moreover, our results support strategies aimed to a rapid reduction of BCR-ABL1 transcript levels to optimize potential suitability for imatinib discontinuation studies.

C093

NON GENOMIC LOSS OF FUNCTION OF TUMOR SUPPRESSORS IN CML: BCR-ABL PROMOTES P53 NUCLEAR EXCLUSION THROUGH THE INTERACTION WITH IKB-ALPHA

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Introduction. The function of tumor suppressors can be tightly controlled by various non-genomic mechanisms, such as epigenetic silencing, regulation by non-coding RNAs and post translational modifications. The identification of mechanisms that cause non genomic loss of function of tumor suppressors could have tremendous consequences from the therapeutic standpoint. Targeting pathways that inactivates tumors suppressors could indeed promote the re-activation of a specific tumor suppressor with dramatic biological consequences. In this work, we show that BCR-ABL interacts with IkBα which in turn promotes p53 nuclear exclusion in Chronic Myeloid Leukemia. *Methods*. HEK293T and HeLa cells transfected with IkBα expression vector alone or in combination with Bcr-Abl expression vector and primary cells collected from Chronic Myeloid Leukemia patients at the diagnosis were analysed by immunofluorescence, immunoprecipitation and western blot in order to evaluate IkBα and p53 protein levels, interactions and cellular compartmentalization. Furthermore, kinase assays have been performed with purified proteins to examine the phosphorylation status of IkBα. *Results*. While assessing the cellular compartmentalization of IkBα in BCR-ABL transfected HeLa cells, we observed that IkBα is expressed mostly in the cytosol, while in parental HeLa cells IkBα showed both cytosolic and nuclear localization. Similarly, primary CD34 positive CML cells are characterized by IkB-alpha expression exclusively in the cytosol. Using BCR-ABL transfected cells, we demonstrate that BCR-ABL physically interacts with IkBα through the BCR portion of the chimeric protein BCR-ABL. *In vitro*, BCR-ABL does not appear to directly phosphorylate IkBα on tyrosine residues. IkBα is known to negatively regulate the NF-kB pathway through the interaction with p65 subunit. Importantly, it was also reported that IkB-alpha can interact with the tumor suppressor p53, with consequent inhibition of some of its functions. Interestingly, here we observed that in CML primary cells p53 is also delocalized into the cytosol. Strikingly, we observed that IkBα is physically bound to both p53 and BCR-ABL in the cytosol of CML cells. *Discussion*. In this work, we demonstrate that BCR-ABL promotes p53 nuclear exclusion through the interaction with IkBα. Our data suggest that expression of BCR-ABL could promote non genomic loss of function of the tumor suppressor p53.

C094

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C095

BCR-ABL INACTIVATES CYTOSOLIC PTEN THROUGH CASEIN KINASE II MEDIATED TAIL PHOSPHORYLATION

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Introduction. PTEN is a tumor suppressor gene that inhibits the PI3K-AKT pathway through the de-phosphorylation of phosphoinositide-3,4,5-triphosphate (PIP3) and that controls genomic stability and proliferation through the interaction with nuclear substrates. Beside the canonic genetic inactivation of PTEN by point mutations and deletions, PTEN is also the paradigm for the non genomic loss of function of tumor suppressors. PTEN is indeed targeted by mechanisms that affect mRNA levels, protein stability, localization and protein activity with dramatic consequences in the regulation of protein activity. The identification of non genetically loss tumor suppressors represents an astonishing opportunity from the therapeutic standpoint, due to the implications that the reactivation of a tumor suppressor could have in cancer cells. Recently, we have shown that BCR-ABL promotes PTEN nuclear exclusion by favoring HAUSP mediated PTEN de-ubiquitination. We linked BCR-ABL/HAUSP/PML/PTEN network to the mechanisms of resistance of CML Stem Cells to Tyrosine Kinase Inhibitors. Here, we show that nuclear exclusion of PTEN is associated with accumulation of PTEN in the cytoplasm of CD34 positive CML cells, where it is functionally inhibited by BCR-ABL/Casein Kinase II mediated PTEN tail phosphorylation. *Methods*. CD34 positive cells collected from CML patients were assessed for PTEN, P-AKT and PIP3 expression by immunofluorescence and western immunoblot. Several PTEN mutants have been generated in GFP-tag and Myc-tag vectors to abrogate regulation by CKII phosphorylation. These vectors were expressed in BCR-ABL-NIH3T3 or 32D cell lines and K562 cells. Cells were then assayed for PTEN activity and apoptosis induction. *Results*. We observed that primary CD34 positive CML cells expressed PTEN in the cytosol but also high levels of PIP3 and P-AKT, therefore suggesting that PTEN could be functionally inhibited in CML. Next, we demonstrate that PTEN is highly phosphorylated by the BCR-ABL substrate Casein Kinase II, which promotes PTEN inactivation by favoring the acquisition of a closed conformation of the protein. Expression of PTEN mutants that lack Casein Kinase II phosphorylation sites promotes apoptosis induction in BCR-ABL positive cells. Similarly, treatment with Casein Kinase inhibitors is associated with the induction of apoptosis of primary CD34 positive CML cells through the reactivation of PTEN. Importantly, we demonstrate that this therapeutic approach promotes apoptosis even in the presence of BCR-ABL-T315I mutation. *Conclusions*. We recently proposed that BCR-ABL functionally inactivates PTEN tumor suppressor by favoring PTEN nuclear exclusion. Here we demonstrate that BCR-ABL is also able to inactivate cytosolic PTEN through CKII mediated phosphorylation. Strategies to target mechanisms that promote PTEN nuclear exclusion or cytosolic PTEN inactivation restore normal PTEN functions with dramatic consequences from the therapeutic standpoint.

C096

BIN1 AND RIN1 MODULATION IN CHRONIC MYELOID LEUKEMIA

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Introduction. The role of Bcr-Abl in the pathogenesis of CML is well established, however the mechanisms involved in progression remain poorly understood. By making use of Drosophila Melanogaster transgenic 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 clathrin coated vesicles. Among them, we focused our attention on Bridging integrator 1 (Bin1) and its activator Rin1, a RAS effector protein. *Methods.* Bin1 and Rin1 expression was measured by qRT-PCR in 82 samples from CML patients and in 10 healthy control samples. Among CML, 34 samples were collected at diagnosis and 45 during the TK inhibitors (TKIs) treatment, 11 of which in Complete Molecular Response (CMR), 17 in Major Molecular Response (MMR), 17 in <MMR and 3 resistant patients. Western Blot (WB) analysis was performed to analyze Bin1 and Rin1 expression in control subjects and CML patients. Bin1 and Rin1 expression was also evaluated by qRT-PCR and WB in K562 and HEK-293 cell lines treated with Imatinib (IM). *Results.* qRT-PCR data indicated that Bin1 expression was significantly down-regulated in CML patients at diagnosis compared to healthy subjects ($p<0.001$). Overall, during TKIs therapy, the transcript levels of Bin1 showed an up-regulation trend from <MMR ($p<0.001$) to MMR ($p<0.001$), to CMR ($p<0.001$) compared with diagnosis. Interesting, Bin1 was also down-regulated in TKIs resistant patients as observed in diagnosis ones. These results demonstrated an indirect correlation between Bin1 and Bcr-Abl transcript expression levels (Figure 1 A and B). Bin1 is activated by Rin1 to promote the formation of endosome structures. Besides, Rin1 interacts with Bcr-Abl stabilizing the fusion protein in CML cell lines. We analyzed Rin1 transcript levels in the same cohort of subjects. Like Bin1, Rin1 expression was down-regulated in CML patients at diagnosis respect to healthy subjects ($p<0.05$), and was up-regulated during TKIs therapy from <MMR ($p<0.05$) to MMR ($p<0.001$), to CMR ($p<0.01$) compared with diagnosis. Moreover, Rin1 remained down-regulated in resistant patients (Figure 1 C). To confirm the qRT-PCR data on CML patients, we analyzed both protein expression by WB analysis and we observed the same trend of transcript levels. To better elucidate the inverse correlation between Bcr-Abl, Bin1 and Rin1, we performed *in vitro* experiments, by IM treatment, in K562 and HEK-293. The results showed an up-regulation of Bin1 and Rin1 in K562 after treatment; we did not observe any variation of their expression in non tumorigenic HEK-293. *Conclusions.* Our results show a significant association between Bin1 and Rin1 expression and clinical phases of CML, suggesting an indirect correlation with Bcr-Abl levels. This study proposes a new deregulated mechanism indicating Bin1 and Rin1 as possible players in the maintenance of the abnormal signaling in CML disease.

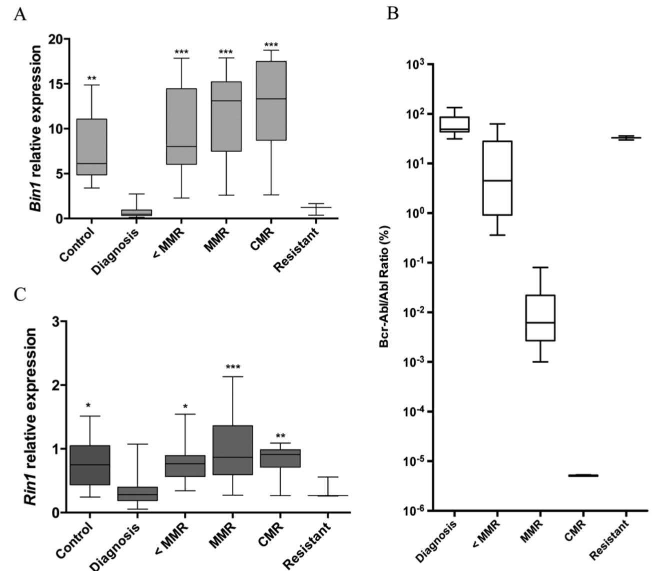

Figure 1.

POSTERS

Stem Cells, Bone Marrow Eicroenvironment

PO-001

AN UNCOMMON CASE OF ACUTE LYMPHOBLASTIC LEUKEMIA OF DONOR ORIGIN IN A PATIENT WITH ACUTE PROMYELOCYTIC LEUKEMIA IN REMISSION AFTER TWO HEMATOPOIETIC STEM CELL TRANSPLANTS

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Introductio. HSCT may be complicated by either disease relapse or development of secondary malignancies. The occurrence of leukemia or MDS from donor cells is recognized as a rare, but probably underestimated complication, with an incidence variable from 0.13% to 5%. *Methods*. A 45-year-old man presented in December 2004 with APL. He achieved hematologic CR with remission induction cycle including ATRA and idarubicin, and subsequently underwent consolidation chemotherapy (CHT). Molecular CR (mCR) was obtained at the end of the treatment in May 2005. Two months later, the patient showed molecular relapse (MR), so that he underwent salvage CHT with ATRA, cytarabine and mitoxantrone, reaching a second mCR. The patient was then treated with an allogeneic PB HSCT from his HLA identical male sibling (donor 1), with myeloablative conditioning. Full donor 1 chimerism was obtained by day +59, as assessed by PCR-based analysis of autosomal short tandem repeats (microsatellites) on DNA samples extracted from BM cells. Unfortunately, in February 2008, the patient experienced another MR, initially treated with DLI and then with ATO, resulting in mCR for the third time. Then, he underwent a second allogeneic transplant from another HLA identical 52-year old male sibling (donor 2), using BM as stem cells source. On this occasion, the conditioning regimen consisted of TBI, CHT and ATG. The patient became a full chimera of donor 2 on day +30, did not experience significant post-transplant complications and had a 5-year disease free interval. On July 2013, fever, asthenia, weight loss and adenopathies occurred. Pancytopenia was found. Either morphologic APL relapse or the occurrence of therapy-related myeloid neoplasm were initially suspected. *Results*. BM aspirate and trephine biopsy examinations demonstrated >90% B-lymphoid blasts. Complex karyotype was observed on cytogenetic analysis. The presence of PML-RARA and BCR-ABL fusion transcripts was excluded. Unexpectedly, the microsatellites analysis showed full chimera donor 2, thus diagnosing B-ALL of donor origin. The patient, refractory to CHT, is actually in CR under treatment with blinatumomab. *Conclusions*. This is the first case of DCL with lymphoid phenotype in a patient with a previous APL diagnosis, after two HSCTs from different male sibling donors, and from different stem cells sources (PB and BM). The causative mechanisms of DCL remain speculative and include sustained antigenic stimulation, defective immune surveillance, replication stress due to proliferation demand, BM irradiation and repeated CHT, leading to an aberrant BM microenvironment. DCL may be under-diagnosed and frequently unexpected in patients observed with the suspicion of leukemia relapse after HSCT. Accurate molecular examinations, especially of chimerism by microsatellites, are essential to investigate the donor origin of leukemic cells, mainly in the cases in which a leukemic cell lineage different from initial diagnosis is found or when more than one HSCT has been performed.

PO-002

IMPACT OF IMPDH1 AND UGT1A9 POLYMORPHISMS ON MYCOPHENOLIC ACID IMMUNOSUPPRESSIVE ACTIVTY IN ALLOGENEIC STEM CELL TRANSPLANTATION

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Introduction. Mycophenolic acid (MPA) inhibits Inosine 5'-monophosphate dehydrogenase (IMPDH) and *de novo* guanine nucleoside synthesis, which is critical for activated T lymphocytes proliferation. MPA is glucuronized in liver by uridine diphosphate-glucuronosyltransferases (UGTs) 1A9 into MPAG, that are excreted into bile, deconiugated into MPA in gut and further reabsorbed via enterohepatic circulation. There are various polymorphisms responsible for activity and metabolism of MPA, such as rs 2278294 and rs2278293 for IMPDH1 and C-440T and T-331C for UGT1A9. *Methods.* We studied the impact of these polymorphisms on allogeneic stem cell transplantation (SCT) outcomes enrolling 60 patients (pts) submitted to SCT in our division, median age 54 ys (range 14-65), 24F/36M. Underlying diseases were: 1 HL, 7 ALL, 6 CLL, 27 AML, 10 NHL, 3 IMF, 4 MM, 2 MDS. Stem cell source was PB in 52 pts, BM in 4 and CB in 4. Two pts received an ablative conditioning while a reduced intensity conditioning was performed in 58 pts. Donor (don) was match related in 38 pts and match unrelated in the other 22 pts. GvHD prophylaxis consisted in CSA and MPA. All couples pts/don were studied for IMPDH1 and UGT1A9 SNPs. Statistical analysis was performed by using IBM SPSS statistic 22. *Results.* Rs294 G/A in pts: 28 GA, 7 AA, 24 GG; don 20 GA, 6 AA, 31 GG. Rs293 G/A pts: 26 GA, 7 AA, 26 GG; don 24 GA, 7 AA, 27 GG. C-440Tpts: 29 CC, 21 CT, 9 TT; don 25 CC, 26 CT, 7 TT. T-331C pts: 27 TT, 24 TC, 8 CC; don 26 TT, 25 TC, 7 CC. Twenty-one pts developed aGVHD at a median time of 25 days (range 1-90) post-SCT. In 22 pts a disease relapse occurred after a median DFS of 4 months (mo) (range 1-40). CMV reactivation was detected in 37 pts with a median viral load of 3425/ml (range 1050-1000000), while in 26 pts a sepsis was identified. At follow up time, 34 pts were dead (median OS 4 mo, range 1-58) while the other 26 were alive with a median OS of 36.5 mo (range 5-95). Pts C-440T CC developed aGvHD earlier (20 days, range 1-58) than others (37.5 days, range 10-90) (p=0.0357). Also in pts T-331C TT aGvHD occurred earlier (17.5 days, range 1-58) than others (35 days, range 10-90) (p=0.036). Among pts with CMV reactivation, who had rs293 GG and GA don showed higher viral load (9293 copies/ml, range 1125-1000000) than others (2340 copies/ml, range 1050-3430) (p=0.0161). Sepsis incidence was higher among pts r294 and r293 GA and AA (20/35 and 19/33) than others (6/24 and 7/26) (p=0.085 and p=0.0215). DFS was longer among pts C-440T TT and T-331C CC (28 mo, range 4-90, and 33.5 mo, range 4-90) compared to others (6.5 mo, range 1-95, and 7 mo, range 1-95) (p=0.0307 and p=0.0406). Finally, pts rs294 GG showed a longer OS (31 mo, range 2-95) compared to others (7 mo, range 1-93)(p=0.0148). (Figure 1). *Conclusions.* IMPDH1 polymorphisms could make itself resistant to inhibition by MPA, allowing T lymphocytes activation and proliferation, while UGT1A9 polymorphisms could reduce MPA catabolism with an extended immunosuppressive effect on T lymphocyte.

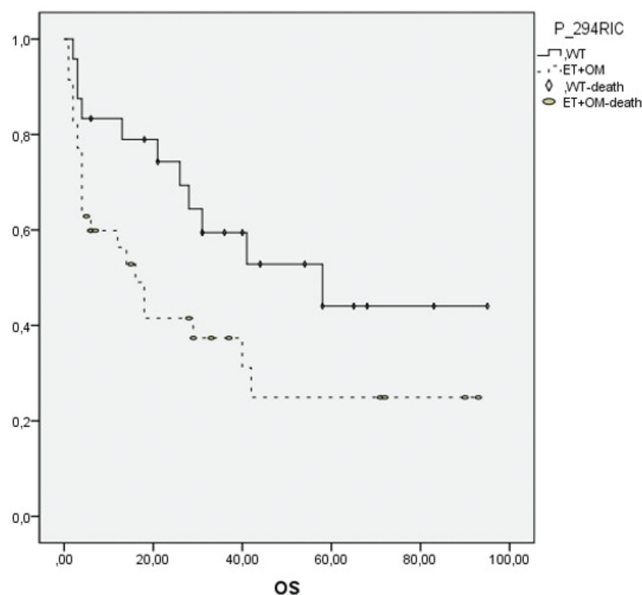

Figure 1.

PO-003

HUMAN HEPARANASE POLYMORPHISMS AFFECTS VIRAL REACTIVATION AND RELAPSE RATE AFTER ALLOGENEIC STEM CELL TRANSPLANTATION

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Introduction. Human Heparanase (HPSE) is a GAG-degrading endoglycosidase responsible for heparan sulphate (HS) degradation and consequent release of HS-bound citokines, chemochines, and angiogenic- and growth-factors. HPSE mRNA, expressed in hematopoietic cells, platelets, and activated endothelial cells, is implicated in various processes such as cell proliferation, angiogenesis, inflammation, tissue repairing and autoimmunity. Recently, two SNPs (rs4693608 and rs4364254) modulating HPSE mRNA level were identified. Some authors reported a correlation between the two SNPs and HPSE mRNA production, defining three groups: GG-CC, GG-CT, GG-TT and GA-CC defined LR (low) group, GA-CT and GA-TT the MR (medium) group and AA-TT and AA-CT the HR (high) group. *Methods.* We evaluate the effect of HPSE genotype on allogeneic stem cell transplantation (SCT) outcome in our division. We enrolled 103 pts, 46F/57M, median age 49 ys (range 10-65). Underlying disease were: AA (1), MM (4), MDS (7), HL (2), ALL (18), AML (59), NHL (9), IMF (3). A reduced intensity conditioning (RIC) was performed in 69 pts while an ablative conditioning (ABL) in 34 pts. Stem cell source was BM in 4 pts, CB in 4 pts and PB in 95 pts. Donor (don) was sibling HLA-match in 59 pts and unrelated HLA-match in 44 pts. HPSE genotype was performed in all but one couple pts/don for SNP rs4693608. Pts: 9 GG, 57 GA and 36 AA. Don: 13 GG, 64 GA and 25 AA. It was possible to perform SNP rs4364254 analysis only in 77 couple pts/don. Pts: 12 CC , 25 CT and 40 TT. Don: 14 CC, 26 CT and 37 TT. Statistical analysis was performed by using IBM SPSS statistic 22.

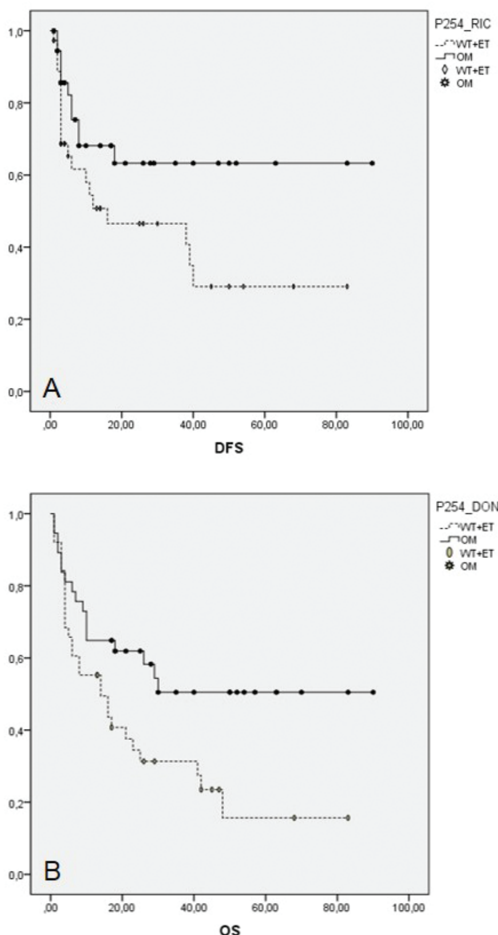

Figure 1.

Results. Forty-two pts developed acute GvHD while chronic GvHD was documented in 43 pts. A disease relapse was documented in 42 pts after median DFS of 4 months (mo) (range 1-40). In 58 pts a CMV reactivation was detected after a median of 40.5 days (range 1-210) post-SCT, whereas 48 pts presented EBV reactivation after a median of 53.5 days (range 4-174) post-SCT. At the follow up time, 64 pts were dead (median OS 6 mo, range 1-48), while 37 pts were alive with median OS of 50 mo (range 13-104). In pts receiving RIC SCT, CMV reactivation rate was higher in LR group (9/9) than in the others (24/42) (p=0.0187). On the contrary, among pts receiving ABL SCT, EBV reactivation rate was higher in SCT performed by LR group don (5/6) than in the others (4/17) (p=0.018). Furthermore, an higher relapse rate was identified in LR/MR groups of pts (20/37) than in the other (11/40) (p=0.0215) with a DFS of 33.996 *vs* 59.360 mo respectively (p=0.049); Figure 1 A. Finally, the OS appeared to correlate with don genotype, with a better outcome (mean OS 51.062 mo) in HR group than the others (mean OS 26.224 mo) (p=0.020). Figure 1 B. *Conclusions.* HPSE was secreted by activated T cells, key cells involved in anti-viral response and GvL. We hypothesized that lower HPSE mRNA production in LR group is associated with impaired control on viral reactivation and MRD after SCT.

PO-004

A PHILADELPHIA CHROMOSOME NEGATIVE ACUTE LYMPHOID LEUKEMIA OF DONOR ORIGIN AFTER UNRELATED ALLOGENEIC BONE MARROW TRANSPLANTATION FOR PHILADELPHIA CHROMOSOME POSITIVE CHRONIC MYELOGENOUS LEUKEMIA

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The development of leukemia in donor cells (DCL) after allogeneic hematopoietic stem cell transplantation (HSCT) is a rare event. In February 2012, patient suffered from LMC Ph+ received HSCT from a male matched unrelated donor.GVHD prophylaxis consisted in cyclosporine-A combined with mycophenolate mofetil. *In vivo* T-cell depletion with rabbit anti-thymocyte globulin was added. By sequence specific primers-HLA typing method, we had typed a DNA samples from the patient before/post HSCT *vs* donor because locus DPB1 was informative (patient DPB1*04:01, donor DPB1*02:01,05:01). Using specific primers and TaqMan probes set, we performed expression profiling in bone marrow (BM) samples before and after HSCT to compare genes and microRNAs (miRNAs) involved in immune/inflammatory networks and hematological differentiation. At day +15 after HSCT, trilineage engraftment was documented by BM examination and patient showed full donor chimerism performed by molecular STR maintaining this result until last follow-up. Six months after HSCT the shift of blood group as the donor was too documented. Also, in peripheral blood and BM, the presence of hybrid transcript b3a2 was determined every month or so by quantitative PCR, with gradual increase until values of 80%, despite TKI treatment. In the same biological samples, standard and fluorescent cytogenetic analysis always indicated male gender (46,XY) and normal karyotype Ph negative. A while after the transplant, we typed a recipient's bone marrow sample for minimal residual disease analysis and locus DPB1. Hybrid transcript b3a2 was higly expressed while locus DPB1 was perfectly identical to the donor's one. Marrow relapse coincided with the recurrence of cerebral acute lymphoblastic leukemia. Immunophenotyping analysis was performed according to the recommendations from the European Group for the Immunological Characterization of Leukemias in multiparameter flow cytometry with CD45 gating. This is the confirmation of our hypothesis of DCL. Comparative analysis of immune response showed increased pro-inflammatory activity in post-HSCT-BM respect to baseline. Three miRNAs (mir150, mir223, mir29a), known for their involvement in the leukemogenesis mechanisms, showed changes in different times, Table 1. One T-test and two way ANOVA were applied considering bone marrow before and after HSCT as variables and all microRNAs as a population of values. After a verification of hypotesis for these 2 tests, they return a p< 0.01 of two variables. *Conclusions*. In our case, the suspect that a female patient developed leukemia from male donor was difficult. In fact, in our patient, use of only one cytogenetic test

could cause diagnostic error due to the loss of the Philadelphia chromosome together to sex-male marker presence and switch of donor blood group. Instead, high sensitive molecular methods such as hematological chimerism and DPI-HLA antigens indicated a strong correlation between the origin and donor leukemia cells.

Table 1. Data are presented as log 2-ΔΔCt of the relative quantity of miRNAs, normalized versus universal RNA, the housekeeping gene was RNU6B. Relative expression level of target genes was based on ΔΔCT method using the ABI PRISM 7900HT Real-Time Sequence Detection System (Applied Biosystems). The 18S pre-developed TaqMan assay (99999901_S1) was used as endogenous control. Samples were normalized versus healthy volunteer BM, housekeeping gene was 18S.

Relative expression level of microRNAs and mRNA-genes		
	Bone marrow before HSCT	Bone marrow after HSCT
Has-mir let7g	4,52	2
Has-mir 103	1,7	2,3
Has-mir 150	7,8	0,001
Has-mir 222	0,56	1,1
Has-mir 223	7,4	34,2
Has-mir 29a	0,04	7,3
IFN γ	12,5	23,8
TNF alpha	155	218
IL-2	34,6	1970
IL 12 A	15	21
IL 18	9	31
IL-6	86	75
IL 4	3	16

PO-005

DEFERASIROX ABROGATES TRASFUSION DEPENDENCE AND CYTOPENIAS IN "POOR GRAFT FUNCTION" PATIENTS AFTER ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Introduction. The attainment of transfusion independence after transplant is sometimes hampered by a combination of factors, ranging from infections to the need of combined therapy for clinical complications, as well as control of GVHD. Iron overload is frequently observed in hematological patients before and after hematopoietic stem cell transplantation. Moreover, iron overload is considered a significant contributor to treatment related mortality. Whereas several reports have focused on iron overload before transplant, little is known on the effects of iron overload on the recovery of hematopoiesis after transplant. All the reports up to now focused on the toxicity correlated to high ferritin level. Our clinical experience for the first time correlated a fast and rapid haematologic recovery with the use of deferasirox in the post transplant setting. *Methods.* we report on 12 patients, transplanted for hematological diseases (11 acute leukemia, 1 aplastic anemia) heavily transfused before transplant, fully engrafted and in complete remission, but still transfusion dependent and with incomplete hematological reconstitution after allo-transplant. No concomitant infection was documented; the work up for other aetiologies resulted negative. Inclusion criteria were: 1) incomplete haematological recovery; 2) more than 20 RBC units before transplant; 3) serum ferritin > 1800 ng/mL; 4) normal creatinine value. All patients received an initial dose of deferasirox 10 mg/kg/day after transplant, later adjusted according to side effects. *Results.* all patients experienced a rapid increase in haemoglobin levels, with a reduction in the frequency of RBC transfusions, followed by transfusion independence (median time: 25 days from the first dose of deferasirox). In addition, the administration of deferasirox was promptly (median time: 27 days) associated with haematological improvement. Moreover, ferritin values progressively reduced with deferasirox treatment. No relevant modifications with immunosuppressive or myelosuppressive drugs were made during deferasirox treatment. Deferasirox was well tolerated. *Conclusions.* Basing on our results, we think that deferasirox determined stimulatory, and/or derepressive effects on hematopoiesis after allo-HSCT. In conclusion, this clinical experience raises the possibility of a potential additive benefit on hematopoiesis after transplant following iron chelation therapy with oral deferasirox. Further long term studies, in larger cohorts of patients are needed to confirm these data and design an efficient strategy to reduce iron loading after transplant. *Acknowledgements.* Supported in part by AIL Pesaro Onlus.

PO-006

INCREASED FERRITIN SERUM LEVEL MAY IMPAIR PERIPHERAL BLOOD STEM CELL COLLECTION IN LYMPHOMA AND MYELOMA PATIENTS UNDERGOING AUTOLOGOUS STEM CELL TRANSPLANTATION

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Background. Iron overload represents an adverse prognostic factor for patients with hematological malignancies undergoing allogeneic stem cell transplantation (SCT). Recent observations have pointed out that pre-transplant ferritin serum level possesses a prognostic value even after autologous stem cell transplantation (ASCT) in multiple myeloma (MM) or lymphoma patients. Aim of the present study was to evaluate whether ferritin serum level, as detected prior to the chemotherapy regimen performed in order to promote PBSC mobilization, has an influence on PBSC collection procedure. *Methods*. The records of all the patients who underwent autologous PBSC transplantation for MM or non-Hodgkin lymphoma (NHL) at our Institution were reviewed; a pre-PBSC collection serum ferritin was available in 38 transfusion -naive patients (21M, 16F, median age=57yrs), 19 MM and 19 NHL. PBSC mobilization regimens included Cyclophosphamide 4g/sqm + G-CSF in all MM patients, while NHL were treated with high-dose cytarabine+ G-CSF (8 patients), ifosfamide- gemcitabine- vinorelbine + G-CSF (8 patients), cytarabine - oxalyplatinum - rituximab (3 patients). *Results*. A ferritin serum level above normal (> 300ug/dl) was observed in 12 patients (8 MM=42% and 4 NHL=21.5%) and was associated with a lower number of collected CD34+ cells x 108/kg (10.9±5.4 *vs* 18.7±13, p=0.05) in a higher number pf apheretic procedures (p=0.04). No correlation was demonstrated with pre transplant disease status and response to therapy, while response duration was shorter (18 *vs* 24.3 months) in patients showing a higher ferritin level, even though the figures did not reach statistical significance due to the low number of patients. *Conclusions*. Our results indicate that an increased serum ferritin level correlates with a reduced PBSC yield in patients undergoing PBSC mobilization, regardless of disease type and mobilization regimen; this observation deserves further investigation in a larger series of patients in order to evaluate the relative role of iron overload, chronic inflammation or other putative mechanisms and to confirm a possible influence on remission duration.

PO-007

FACTORS AFFECTING OUTCOME OF ALLOGENEIC STEM CELL TRANSPLANTATION AS SALVAGE THERAPY IN PATIENTS WITH ACUTE MYELOID LEUKEMIA PRIMARY REFRACTORY TO INTENSIVE INDUCTION CHEMOTHERAPY

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The prognosis of patients with acute myeloid leukemia (AML) refractory to induction chemotherapy is extremely poor. Initial salvage with allogeneic stem cell transplantation (SCT) seems to be associated with superior overall survival (OS) compared to further chemotherapy, particularly in patients receiving intensive induction regimens. Few data regard-

ing factors associated with superior outcome are available in this setting. We reviewed our database and identified 34 patients with AML primary refractory after intensive induction chemotherapy who underwent allogeneic SCT at the Division of Hematology of Udine between 2000 and 2013. All patients received an induction chemotherapy with high dose cytarabine (HDAC) and idarubicin, associated in 31 cases with fludarabine (n=27) or etoposide (n=4). Twenty-seven patients received at least a second course of chemotherapy with HDAC and idarubicin, without achieving complete remission (CR). Median number of chemotherapy courses before SCT was 2 (range, 1-4). Median age at SCT was 55 years (range, 27-69), and SCT was performed at a median of 5.5 months (range, 1.6-10) from diagnosis. Twenty-one patients (62%) received grafts from a sibling donor and 13 (38%) from a matched unrelated donor. In 27 patients (79%) stem cells source was peripheral blood (PB), while bone marrow (BM) stem cells were used in 7 cases. Patients were conditioned either with myeloablative (n=18) or reduced-intensity conditioning (n=16) regimens. OS was defined as time from SCT to death or last follow-up. Twenty patients (59%) achieved CR after SCT, but 14/20 (70%) relapsed, after a median of 3.4 months (range, 1.7-18.2) from CR. Thirty patients (88%) died, with a median OS of 4.5 months (95%CI 2.6-8.6) and a 3-year OS rate of 18% (95%CI 7-32). Cause of death was disease relapse/progression in 22 cases (73%). OS was not predicted by gender, karyotype, age at SCT, time to SCT, number of chemotherapy courses, donor type, stem cell source or conditioning regimen. There was a significant association for WBC count before SCT (<10000/ml: 20 months [95%CI 4.6-NA] *vs* ≥10000/ml: 3.6 months [95%CI 1.9-4.5], p<0.0001), platelet (PLT) count before SCT (>30000/ml: 18.8 months [95%CI 3.6-NA] *vs* ≤30000/ml: 3.7 months [95%C 1.9-10], p=0.05) and circulating blasts before SCT (0: 79 months [95%CI 2.6-NA] *vs* ≥1: 4.5 months [95%C 3.4-8.6], p=0.03). Stratifying patients according to WBC, PLT and circulating blasts, we identified four groups with different survival: median OS for patients with 0, 1,2 or 3 adverse features was 78, 10.3, 4.1 and 3.5 months, respectively (p=0.0009) (Figurre 1). Our data confirm that allogeneic SCT can cure a minority of patients with primary refractory AML, that relapse is the major cause of treatment failure after SCT and that pre-transplant WBC and platelet counts and circulating blasts may predict long-term outcome.

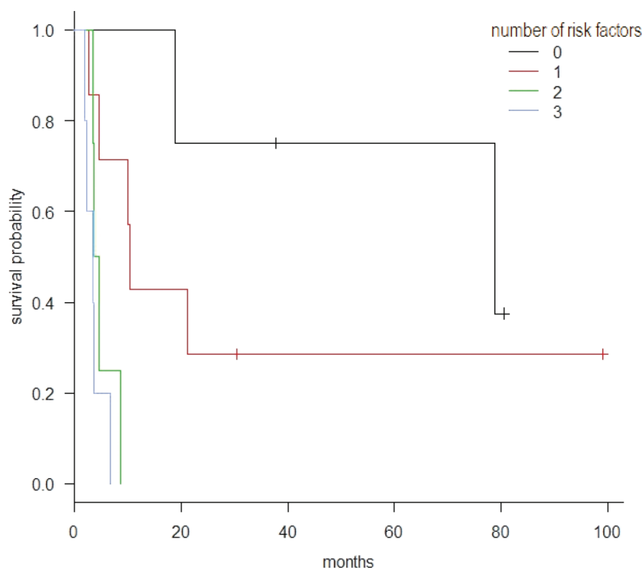

Figure 1.

PO-008

PROGNOSTIC VALUE OF MINIMAL RESIDUAL DISEASE BEFORE ALLOGENEIC MARROW TRANSPLANTATION IN ACUTE MYELOID LEUKEMIA BY COMBINED WT1 EXPRESSION LEVELS AND FLOW CYTOMETRY ASSESSMENT

Guolo F, Minetto P, Galaverna F, Giannoni L, Clavio M, Di Grazia C, Grasso R, Colombo N, Avenoso D, Pastori G, Ballerini F, Miglino M, Kunkl A, Lemoli RM, Gobbi M, Bacigalupo A

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Background and Aims. Allogeneic bone marrow transplantation (BMT) offersthe greatest chance of cure for most patients affected by acute myeloid leukemia (AML). Persistence of disease or high levels of pre BMT minimal residual disease (MRD) have been reported to predict disease relapse after BMT.WT1 expression levels and multicolor flow cytometry (MFC) are widely used as markers of MRD.We recently reported that combined evaluationof MRD by WT1 andFCafter induction therapy has a strong impact on relapse risk in AML patients.The aim of the present study was to apply the same MRD assessment in pre BMT setting to evaluate its reliability in predicting relapse. *Materials and Methods.* We retrospectively analyzed BMT outcome of 66 AML patients with both WT1-based and MFC-based MRD evaluation on bone marrow samples before transplant. Median age at transplant was 44 years. Forty-two patients were transplanted in first and 24 in second or subsequent complete remission. Induction regimens included fludarabine-containing regimens or standard “3+7” induction. Median follow-up was 24 months (range 1-117 months). Disease-free survival (DFS) was calculated from the time of transplantation until last follow-up or documented leukemic relapse. A positive MFC MRD was defined by the presence of no less than 25 clustered leukemic cells /10^5 total events (threshold of 2.5x10^-4 residual leukemic cells) at four-color flow-cytometry. Real-time PCR for WT1 was performed on DNA Engine 2 (Opticon®, MJ Research®). WT1 copy number/Abl copy number 1000x10^4 was used as cut-off value for high WT1 expression. *Results.* Twenty-five relapses (37.9%) were observed. Median DFS was 31 months. Our preliminary results show that the probability of disease relapse was significantly influenced only by disease status (first or subsequent CR) and MRD status at transplantation. Specifically, MFC-MRD was the strongest predictor of longer disease free survival (p <0.001) since no relapses occurred in the eleven MFC-MRD negative patients. Among MFC-MRD positive patients a further stratification of risk is obtained by the evaluation of WT1 MRD status that was able to identify patients with significantly worse DFS. (p <0.01, Figure 1).The predictive value of MRD resulted independent from different induction schedules; furthermore undergoing BMT in second or subsequent remission did not affect the positive prognostic value of gaining a negative MRD status. *Conclusions.* Pre BMT evaluation of MRD by WT1 and MFC on bone marrow samples is a reliable predictor of relapse risk. Patients with negative pre-BMT MRD have a significantly longer DFS, while patients with both MRD markers display an higher risk of relapse. Identifying patients at higher risk may allow to modulate post BMT follow up, to detect earlier disease recurrence and perhaps to apply pre-emptive therapeutic strategies in order to delay or avoid AML relapse.

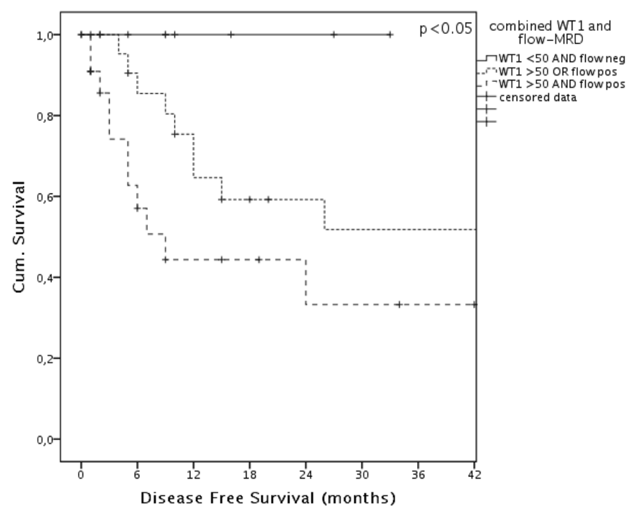

Figure 1.

PO-009

FUNCTIONAL *IN VITRO* STUDIES OF BUFFY COAT POOLED PLATELETS CRYOPRESERVED WITH A INNOVATIVE METHOD UP TO NINE MONTHS

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Introduction. Cryopreservation for long term storage of platelets (PLTs) represents a clinical useful method for avoiding platelet shortage. We have performed an *in vitro* prospective study to assay buffy coat derived pooled platelet concentrates (BC-PLTs) treated with dimethyl-sulphoxide (DMSO) and cryopreserved at -80°C up to nine months with an innovative patented system avoiding laminal flow hoods and external manipulations. *Materials and Methods*. Each BC-PLTs was obtained from 5 buffy coats. PLTs concentrates were leukoreduced by filtration and transferred to a 650 mL cryopreservation kit (Promedical®) which allowed mixing with DMSO 25% in a closed system and supernatant removal without further manipulations. BC-PLTs were analyzed immediately pre-freezing (T0) and up to 9 months after cryopreservation (CRY BC-PLTs). The following parameters were assayed: PLTs count (PC), mean platelet volume (MPV), pH, flow cytometry (FACS) expression of CD41a, CD42b, CD61a, CD62p, PAC-1, Annexin V PLTs surface antigens and thromboelastography (TEG). All samples were analyzed also after dilution (1:4) with homologous plasma to approximately 400 x109/L PLTs (data not shown) . All the tests were performed according to current European recommendations. PLTs swirl was furthermore visually assessed. Results were expressed as mean +/- standard deviation (SD). Results obtained at T0 and after 9 months were compared by paired sample t-test. Differences were considered as significant at p values <0.05. *Result*. *In vitro* cell parameters were measured on 49 BC-PLTs and CRY BC-PLTs at 3 (T3), 6 (T6) and 9 (T9) months respectively as reported in Table1. PC was only slightly reduced in CRY BC-PLTs while MPV was significantly increased in CRY BC-PLTs. There were no differences between groups in CD41a, CD61a, CD62p and Annexin V expression while a significant reduction in CD 42b, PAC-1 for CRY BC-PLTs was observed. TEG parameters were all significantly reduced in CRY BC-PLTs samples without affecting hemostasis. PLTs swirl was observed in all samples and BC was absent.All the analyzed parameters showed stable values during cryopreservation (Table 1). There were no differences between the groups in CD41a, CD62p and Annexin V expression while a significant reduction in CD 42b , PAC-1 for CRY BC-PLTs was observed after cryoprserevation . TEG parameters were all significantly reduced in CRY BC-PLTs samples, not affecting hemostatic capacities. *Conclusions*. Current results confirm the potential, to be confirmed *in vivo*, of a new system to overcome limits to PLTs storage. In fact, this method guarantees sterility and avoids excessive manipulations thanks to a closed system. Our method avoids PLTs apoptosis, as shown by absent expression of Annexin V. The adequate hemostasis achieved at TEG in both groups supports the hypothesis that *in vitro* PLTs activation/deterioration doesn't necessarily mirror an impaired hemostatic *in vivo* function of CRY BC-PLTs.

Table 1. *In vitro* assays of pre-freeze and cryopreserved platelets.

	Pre-freeze (n= 49)	Cryopreserved at 3 months	Cryopreserved at 6 months	Cryopreserved at 9 months
Platelet count ($x10^9$/L)	1427 +/- 150	1335 +/- 99.34	1290 +/- 88.4	1200 +/- 78.4
MPV (fL)	8.6 +/- 0.43	12.01 +/- 0.5*	11.9 +/- 0.6*	12.1 +/- 0.4*
pH	7.3 +/- 0.07	7.1 +/- 0.1	7.2 +/- 0.1	7.3 +/- 0.1
GPIb/CD42b (%)	92.7 +/- 4.29	23.6 +/- 27.5*	16.38+/- 12.54*	17.3 +/- 9.6*
GPIIb/CD41a(%)	98.5 +/- 1.94	98.1 +/- 3.07	98.3 +/- 1.24	97.96 +/- 3.1
GP53/CD62p (%)	59.0 +/- 11.02	71.1 +/- 14.6	76.89 +/- 8.65	70.9 +/- 7.4
PAC-1 (%)	1.9 +/- 1.34	0.62 +/- 0.4*	0.63 +/- 0.83 *	0.49 +/- 0.48*
Annexin-V (%)	0	0.03 +/- 0.04	0.03 +/- 0.04	0.01 +/- 0.02
TEG r (min)	8.3 +/- 2.55	10.85+/- 1.6*	11.03+/- 2.69*	9.5+/- 1.89*
TEG k (min)	1.6 +/- 0.3	2.96+/- 1.04*	3,2.9 +/- 1.3*	2.8 +/-0.6*
TEG a (deg)	71.9 +/- 10.7	53.4 + /- 9.5*	52.11 +/- 13.9*	57.6 +/- 11.0*
TEG MA (mm)	70.61 +/- 10	62.3+/- 8.79*	62.4 +/- 7.86*	60.9 +/- 7.71*
TEG ly30 (%)	11.84 +/- 17.07	10.85+/- 1.6*	11.03+/- 2.69 *	9.5+/- 1.89*

Values shown are mean +/- SD

*Indicates p < 0.05 compared to pre-freeze

PO-010

PRO-BNP AND hs-TROPONINE T RELATE WITH ECHOCARDIOGRAPHIC CHANGES IN THALASSEMIC PATIENTS

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Background. Myocardial iron overload is the main cause of Heart failure (HF) in β-thalassemia major (β-TM) patients. A prompt diagnosis of HF is crucial in determining the survival of these patients. We aimed to evaluate myocardial impairment dosing the levels of high sensitive-troponine-T (hs-TnT) and pro-BNP (pro-Brain Natriuretic Factor) in serum of β-TM patients relating these findings with iron overload indices and echocardiographic measurements. *Methods*. This pilot study recruited 50 consecutive β-TM patients from June 2013 to January 2014 at Our Institution. Male/Female ratio was 1:1; Median age was 32 years (range 16-48). All of them were affected by β-TM, except 2 male patients affected by intermediate Thalassemia with Major-like phenotype. Echocardiographic measurements were carried out according to the recommendations of the American Society of Echocardiography. All indexes were referred to the Body Surface Area (BSA). HF was defined if clinically evident signs or symptoms were present. Informed consent was given to participants. *Results*. Information on LVM was available for 46 out of 50 patients. All patients presented a normal left ventricular ejection fraction (LVEF). Median value of TnT was 3,925 ng/L, ranged between 3 and 60,3 (normal range 0-14). Median value of pro-BNP was 56,8 ranged between 3,99 and 1415 pg/mL (normal values 0-125). Overall, 4 patients presented levels of hs-TnT greater than 14 and 13 patients presented levels of pro-BNP greater than 125. 1 patient presented augmented levels of both pro-BNP and hs-TnT. 6 patients presented HF, 3 with normal levels of pro-BNP and hs-TnT, 2 patients with augmented levels of pro-BNP and 1 patient with augmented levels of both pro-BNP and hs-TnT. Hs-TnT related with LVMi (Left Ventricular Mass indexed to BSA) (Correlation coefficient r:0,7; p=0,01; 95%CI for r: 0,1455-1,257). Pro-BNP levels related with the LAVi (Left Atrial Volume indexed) (Correlation coefficient r:0,03975; p=0,0051; 95%CI for r:0,01-0,06). Median values for LVMi and LAVi were respectively 83,7 g/m2(range 42-125) and 26,2 mL/m2 (range 11,7-61,8). Additionally, the presence of an HF related with higher levels of hs-TnT with a cutoff value of 4,23 ng/L (sensitivity:100%, specificity:65,12%; AUC:0,7; p<0,01) but less with pro-BNP with a cutoff value of 140,7 pg/L (sensitivity:100%, specificity:65,12%; AUC:0,6; p=0,4457). No relationships were found with iron overload markers (Ferritin; Saturation Index; T2* of the Liver and Heart) neither with the average Haemoglobin Level of the patient and the Ejection Fraction at Echography. *Conclusions*. Taken together, our findings suggest that, in β-TM patients, hs-TnT and pro-BNP could be sensitive indices of myocardial impairment with preserved LVEF.

PO-011

INCIDENCE AND MANAGEMENT OF VENOUS THROMBOSIS IN ACUTE LEUKEMIA: A MULTICENTER STUDY

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Introduction. Venous thrombosis (VT) frequently complicates the clinical course of cancer. We have performed a multicenter retrospective study with the primary objective to evaluate the incidence of venous thrombotic complications in a population of patients with AL. Second-

ary objective was to evaluate the management of these complications. *Materials and Methods*. Available clinical records of out and in-patients diagnosed with AL from January 2008 to June 2013 in 7 Regional Reference Hospitals were analyzed. Cases of VT, including thrombosis in atypical sites [Retinal occlusion (RO) and Cerebral Sinus Thrombosis (CST)], were reported in the current study. Available laboratory tests at diagnosis of VT included complete blood cells count (CBC), basal coagulation tests (PT, aPTT, fibrinogen), Antithrombin, anticoagulant Protein S and C and D-dimer. Instrumental diagnosis was performed according to ACCP guidelines. In the statistical analysis, logistic regression model was applied. *Results*. Over a population of 1461 patients with AL, 99 cases of VT were recorded, mainly in hospitalized patients: 72 cases were associated with Acute Myeloid Leukemia (AML) and 27 with Acute Lymphoblastic Leukemia (ALL). In the cohort of patients with VT,56 were males and 43 females , with a mean age of 54.7±13,1 years. Twelve patients were receiving anticoagulant prophylactic treatment with low molecular weight heparin (LMWH) at diagnosis. There were 74 cases of DVT of upper arms, 14 cases of proximal DVT of limbs (3 complicated with PE), 3 cases of RO, 2 of CST and 1 case of intracardiac clot. In 78/99 (78,8%) cases of recorded VT, a central venous catheter (CVC) was placed; moreover, 68/78 events of DVT of upper arms were significantly associated with a CVC insertion ($p< 0.01$). VT occurred during chemotherapy (CHT) in 90/99 (90%) cases. At CBC, thrombocytopenia was the most frequently observed laboratory abnormality. Coagulation tests were normal in all cases. Most VT episodes (87/99) were treated with LMWH at therapeutic doses for the first month after diagnosis, a dose reduction was recorded in the following months, mainly related to severe thrombocytopenia after CHT. No cases of VT–related deaths nor fatal complications during treatment for VT were recorded. Treatments with LMWH lasted from 3 to 6 months. All patients clinically recovered from VT, only 2 late recurrences (PEs) were observed. *Conclusions*. The incidence (6.7%) of VT in the analyzed cohort of patients with AL is almost similar to only one previous report, even if the involved sites distribution appears quite different. In particular, RO has never been reported. Atypical sites VT must be suspected to be correctly diagnosed and treated. The optimal management of VT in patients with AL requires further, prospective studies.

PO-012

MANAGEMENT OF POST-CHEMOTHERAPY NEUTROPENIA IN RELAPSED AND REFRACTORY MULTIPLE MYELOMA: ROLE OF PEGFILGRASTIM

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Pegfilgrastim is a pegylated long-acting recombinant form of G-CSF that extends the half-life and allows for once-per-cycle dosing, requiring less frequent dosing than nonpegylated G-CSF. Multiple Myeloma (MM) in advanced phases may be managed by regimens combining agents not frequently employed in early phases of treatment, but myelotoxicity is the main expected side effect and G-CSFs are often necessary to counteract the risks of febrile neutropenia. Our aim was to compare the efficacy and safety of pegfilgrastim in patients affected by relapsed/refractory MM. In order to determine whether a single subcutaneous injection of pegfilgrastim is as effective as daily injections of standard filgrastim, in terms of haematological toxicity, febrile neutropenic episodes, antibiotic usage and hospedalization duration. We enrolled in our study 29 patients (16 male and 13 female) with a median age of 64.7 years (range 39-82) affected by multiple myeloma, all relapsed and refractory to a median of 6.3 lines of therapy (range 4-8). Since first course, received in our out-patient department, patients performed blood counts twice weekly and received, from day +8 to day +19 (considering "day + 1" the day in which the chemotherapy protocol starts), prophylactic oral chinolonic antibiotics and anti-fungal drugs. During neutropenia after first cycle of chemotherapy, Filgrastim (5 μgr/kg/day for 3 days) was given if neutrophils count was <1500 x 10^9 cells/L. Median number of filgrastim administrations was 4.7 (r. 3-6); nadir neutropenia was registered after a median of 11.3 days (r. 8-14); median of nadir neutrophil count was 1.16 x 10^9 cells/L (range 0.4 – 1.8 x 10^9 cells/L), with maximum duration of 13 days. From the second course of chemotherapy, all patients switched to prophylactic therapy with pegfilgrastim (6 mg), injected subcutaneously with a single administration on day +3 independently from the neutrophil count at that time. Primary endpoint was the duration of neutropenia (N< 1.5 x 10^9 cells/L), comparing pegfilgrastim and filgrastim. During pegfilgrastim, neutropenia was never longer than 8 days, with a consequent reduction of neutropenia-related infections. Median nadir neutrophil count, evaluated for every patients for at least three courses of therapy (r. 3-6) registered at day +11, was 1.628 (range 0.93-2.25 x 10^9 cells/L); only four patients (13.7%) needed, one week after pegfilgrastim administration, a supplement of 3 administrations of filgrastim. During pegfilgrastim prophylaxis, neutropenia was shorter than during Filgrastim treatment. Besides the mono-administration, pegfilgrastim was well tolerated in all patients: main side effects in our patients were mild fever and bone pain, (5/29 patients,17%). In conclusions, in patients affected by MM exposed to myelosuppressive agents in advanced phases of MM, pegfilgrastim seems to reduce the incidence of neutropenia and may increase the possibility to maintain the scheduled time of treatment.

PO-013

MANAGEMENT OF MYELODYSPLASTIC SYNDROMES WITH ERYTHROPOIESIS STIMULATING AGENTS: EVALUATION OF ERYTHROPOIETIC ASPECTS AND ANALYSIS OF RESPONSE

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Erythropoiesis stimulating agents (ESAs) are the frontline treatment in low risk anemic MDS patients and an employment of this therapy in the earlier stage of the disease can delay the need for RBC transfusion, hypothetically by slowing the disease course. It is a matter of debate whether the clinical response is a result of proliferation and maturation of the dysplastic clone or stimulation of residual normal erythropoiesis by ESAs. Evidence has been provided to support both views. Macrocytosis is one of the cytological hallmarks of dyserithropoiesis in MDS: we have analyzed clinical and erythropoietic response to ESAs therapy in a cohort of anemic not trasfusion-dependent MDS patients, enrolled in a retrospective register, RECAMDS, a subgroup of the Italian MDS register. We focused on clinical response and cytometric differences in MCV during the observation period in order to speculate on the target of such therapy in responsive patients, with a sub analysis of the patients according to WHO and IPSS risk stratification. We have retrospectively analyzed 124 anemic MDS patients (M/F 60/64, median age 78 y.o., WHO subgroups: 44 RA, 19 RARS, 47 RCMD, 6 RAEB-1, 2 RAEB-2, 6 MDS del5q) not transfusion dependent, under standard ESA treatment (α or β EPO 40000/80000 or 30000/60000 U/W respectively), from 2006 to 2014. The data analysis was performed at the baseline, after three and six month of continuous therapy, according to IWG criteria 2006. ESA therapy was started at mean Hb concentration of 9.5 g/dl±1.5, mean serum EPO concentration: 63.2 mU/L, after a mean time from diagnosis of six months (r.1-118). Overall response rate (ORR) was 84% (104/124) and no difference among WHO subgroups was found. 114 were the responders: 96 patients responded after three months, 8 after six. In the responsive patients, at baseline, 57/104 (54%) patients were macrocytic and 46/104 (44%) were normocytic, while, in the group of the 20 non-responsive patient, 17/20 (85%) exhibited macrocytosis at baseline. Then, in the same subgroup of responders, after 6 months from beginning of ESAs treatment 42/57 (73%) macrocytic patients showed permanently elevated values of MCV whereas 12/57 (21%) macrocytic responsive patients became permanently normocytic. Moreover, considering the group of 46 responsive patients which were normocytic at baseline, 13/46 (28%) became macrocytic at 6 months and 4 of these showed a contemporary increase in their neutropenia and/or thrombocytopenia, regarded as first signs of progression of disease. These very preliminary data can suggest that in the majority of MDS patients responsive to ESA treatment the increase of hemoglobin level occurs mainly stimulating erythroid production in MDS clones; in the minority of patients probably it happens recruiting residual polyclonal erythropoiesis. It is interesting to note that stimulating effects of ESA last even when the expression of dysplasia progresses.

Molecular and Cytogenetic Alterations

PO-014

ARGONAUTE-2 AS NOVEL MOLECULAR TARGET FOR THE DIFFERENTIATION THERAPY OF ACUTE MYELOID LEUKEMIA CELLS

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Introduction. In hematopoietic stem cells, growth and maturation of erythroid, granulocytic, monocytic and megakaryocytic lineages are largely controlled by unique combinations of transcription factors that cooperatively regulate promoters and enhancers present on specific target genes. miRNAs provide an additional level of control beyond the transcription factors and play a central role in hematopoietic differentiation through the establishment of complex regulatory circuitries. These small RNAs, to exert their function, are assembled in the functional RNA-induced silencing complexes (RISCs), where a member of Argonaute (Ago) family of proteins, Ago1-4, provides a unique platform for target recognition and gene silencing. Alteration of miRNAs levels and functional activity may affect proliferation, differentiation and genetic stability of hematopoietic stem/progenitor cells (HPCs), resulting in myeloproliferative disorders and leukaemia. *Methods.* By using myeloid cell lines and primary blasts we highlight Ago2 as a new player in myeloid cell fate determination. By Western-blot analysis we observed that Ago2 protein levels are increased during monocyte differentiation of HL60 and Monomac-6 cell lines, whereas are down-regulated during granulocyte differentiation of NB4 cell line and freshly isolated APL primary blasts. Of note the shRNA-mediated down-regulation of Ago2 impairs the monocityc differentiation of AML cell lines whereas improves the Retinoic Acid (RA) differentiation response of the APL cell line NB4. Thanks to the availability of the crystallographic structure of the human full-length Ago2, by means of high-throughput docking screening, we selected a small-molecule targeting the miRNA binding domain of Ago2 able to bind Ago2 and to inhibit Ago2-miRNAs interaction. *Results.* We observed that treatment of the APL cell line NB4 with the small molecule we selected (at a final concentration of 0.1 e 10 μM) was able to increase RA-dependent granulocytic differentiation, evaluated by morphological and functional analysis (evaluation of phagocytic and cytotoxic activity by the NBT assay), with effects similar to Ago2 shRNA-mediated down-regulation. *Conclusions*. The identification of small molecules that do not need to be delivered to the cell, with the ability to functionally inhibit Ago2, pave the way for studies to dissect Ago2 involvement in hematopoietic differentiation and more importantly to improve the RA differentiation response also in other non-APL AML subtypes.

PO-015

CHARACTERIZATION OF THE UNFOLDED PROTEIN RESPONSE ROLE IN DIFFERENTIATION THERAPY OF ACUTE MYELOID LEUKEMIAS

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Introduction. Acute myeloid leukemia (AML) is caused by the clonal expansion of hematopoietic myeloid precursors blocked at different stages of differentiation. A subtype of AML, acute promyelocytic leukemia (APL), is a paradigm of differentiation therapy since all-trans-retinoic acid (ATRA)-based treatments are able to induce leukemic blast terminal differentiation, leading to clinical remission in the majority of APL patients. However, ATRA can lead to systemic toxicity and relapses after initial remission followed by resistance. Furthermore APL accounts for about 10-15% of AML cases and non-APL AML respond only very slightly to ATRA. Thus the search for a strategy to further sensitize AML cells to ATRA is highly needed. ATRA induces differentiation of APL blasts to granulocytes that are secretory cells since they are characterized by the presence of secretory granules containing peptides indispensable for their role in the immune response. The majority of proteins secreted or resident on the plasma membrane are folded and assembled in the endoplasmic reticulum (ER) which, to cope with the burden of folding proteins, activates a series of intracellular signal transduction pathways, collectively named the unfolded protein response (UPR). The UPR intervenes in reliving ER stress by temporarily attenuating translation and promoting expansion of the secretory apparatus; however, if the stress is too prolonged the UPR triggers pro-apoptotic pathways. We set out to investigate if the UPR plays a role in ATRA-dependent AML differentiation and to exploit ER stress to sensitize AML cells to ATRA. *Methods.* We followed RA-induced differentiation of the human promyelocytic cell line NB4 and of the myeloblastic cell line HL60, in the presence or in the absence of ER stress, evaluating cell viability (by propidium iodide exclusion assay), cell differentiation (by morphological modifications and expression of granulocyte-specific genes) and activation of the UPR (by real-time PCR and Western Blot). *Results*. We identified increased expression and/or activation of elements of the UPR in ATRA-treated AML human cell lines. Importantly induction of ER stress in ATRA-treated AML cells determines a more differentiated morphology and a higher rate of cell death with respect to cells not induced to differentiate by ATRA. *Conclusions*. We verified the activation of some UPR components in ATRA-dependent AML cell differentiation and found that differentiating AML cells are more sensitive to ER stress-induced cell death. Our findings could help designing new strategies to further sensitize leukemic cells to ATRA.

PO-016

MOLECULAR EVIDENCES FOR AN ALTERNATIVE STRUCTURE OF WT1 EXON 1 ARISING FROM A REPEAT ELEMENT INSERTION

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Introduction. The role of WT1 gene in acute leukemia pathogenesis is still partially unclear; it may act both as tumor suppressor and as oncogene. It is highly expressed in several hematopoietic malignancies, including AML and has a significant role in MRD and evaluation of cancer progression/relapse.[1,2] A wide number of WT1 mutations was identified in the last years, in particular within exons 7 and 9. The majority of these variations are responsible for an altered gene regulation.[3] Another region significantly involved in gene regulation is the WT1 promoter: it is also target for epigenetic control, such as methylation. Hypermethylation of CpG islands leads to the lack of WT1 expression.[4] In this study we aim to investigate the WT1 mutational state with particular focus to its promoter. *Methods.* Bioinformatics and molecular analysis were performed by applying: a) primer design for detecting all WT1 regions (NCBI Primer-Blast); b) DNA isolation from bone marrow of 9 patients at AML onset and 3 controls coming from Basilicata area (South-Italy) by Nucleosplin Blood Kit (Macherey-Nagel); c) WT1 amplification by PCR (AmpliTaq Gold-DNAPolymerase kit, Ambion); d) fragment sequencing and e) computational biology for DNA variant analysis (BlastN tool and Mutation Surveyor software). *Results.* We identified in all patients a novel "non canonical" WT1 exon 1 structure: the insertion of a 341 bp fragment near the promoter. By investigating the inserted element we notified that it shows identity to a repeat region, such as a MIR region, belonging to the short interspersed nuclear element (SINE) family. (Figure 1). SINE elements correspond to the non-coding DNA with regulation role and they are localized at level of transcriptional unity, preferentially within the C-G rich regions.[5,6] In our case, the transcriptional events could be modulated by the presence of the repeat element. Besides this direct role in leukemia pathogenesis, we hypothesize also an indirect role, by affecting WT1 network, in particular referred to the genes involved in methylation such as methyltransferases (DNMT3). In fact, it has been recently demonstrated that SINE repeats are enriched around the transcription start sites of gene that are rarely or never methylated in cancer.[5] In addition, we suggest a founder effect with propagation of the alternative WT1 exon 1 in Basilicata region. *Conclusions.* We reported a non previously described structure of WT1 exon deriving from the insertion of a repeat element (SINE element). It could affect the gene transcription and, consequently, the gene regulation pathway leading to an uncontrolled gene product production, also related to a lack of methylation.

This aspect is consistent with the aggressive leukemia course observed in our patients.

Figure 1.

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PO-017

FAST AND NON-INVASIVE IDENTIFICATION OF TUMORIGENIC-PROLIFERATIVE MOLECULAR BIOMARKERS IN LYMPHOPROLIFERATIVE DISORDERS

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Monoclonal gammopathy (MG) also affects the elderly population and its etiology remains unknown. Diagnostic criteria for MG of undetermined significance (MGUS) requires quantification of bone marrow plasma cells and radiologic procedures. Polycythemia vera (PV) and essential thrombocythemia (ET) are clonal diseases (MPD) and may develop common features including a small potential for transforming into acute leukemia. Concomitant cases of MPD with MG have been described in the literature (MPD/MG). To measure relative expression of potential pathogenetic genes involved in apoptotic control, stem-cell differentiation, immune network, inflammation and leukemogenesis, in order to determine whether these may serve as biomarkers to better discriminate a subset of lymphoproliferative or myeloproliferative hematological disease without performing bone marrow aspirate. Global transcriptional profiling studies were performed using peripheral blood samples (PBMCs) from MG, MPD/MG and myelofibrosis (M) patients. PBMCs from 16 healthy donors (Group 1) were used as controls. Patients were divided in groups: MPD/MG (Group 2, n=8), MG (Group 3, n=8), M (Group 4, n=9). A multigene expression assay (47 genes) was carried out with the TaqMan® Low Density Array Fluidic card. In this study, we observed 6 differentially expressed genes (BMI-1, FLT3, FZD1, FZD5, ICAM1, IMP3) in Group 3 (MG). We compared variance, main value for each gene in each group and T-test showed a significant differential expression pattern ($p<0.01$) for Group 3 with respect to other Groups for each of these 6 genes. Moreover, we attempted a complete class separation by applying the Support Vector Machine statistical learning algorithm with a linear kernel. Class separation is highlighted in Fig. 1. Group 3(MG) was completely separated from other groups MPD/MG, M and from normal subjects. In Group 3(MG), all 6 genes were over-expressed respect to the normal group. These results describe the altered expression of a subset of genes including BMI-1 (polycomb ring finger oncogene) in PBMCs samples from patients suffering from GM. Of note, we were unable to identify any specific markers in myeloproliferative disorders evaluated in parallel. We propose that the prospective screening for BMI-1 expression alone or in combination with other molecular markers may help refine disease staging and prognosis towards the optimization of therapeutic interventions. Our data indicate that the evaluation of the BMI1gene expression in total PBMCs may represent a more practical diagnostic test using more easily obtainable and less expensive biologic material, thus avoiding more invasive procedures in patients. . It is noteworthy that over-expression of BMI-1was reported in U266 myeloma cells and it has been proposed as a key regulator of cell growth control/senescence in tumorigenesis and lymphomagenesis. Other genes such as FZD1-FDZ5 have been reported to be involved in dysregulation of B-cell leukemogenesis.

Figure 1. We applied a SVM classification simulation with a linear kernel regression on a sub dataset with the six genes identified. It is easy to make a good class separation for Group 3 (Monoclonal Gammopathy.) *vs* other groups (MPD/MG) and M.

PO-018

RAFV600E MUTATION DETECTION IN HAIRY CELL LEUKEMIA BY THE DIGITAL PCR

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Hairy cell leukemia (HCL), that represents approximately 2% of all leukemias, is an indolent lymphoproliferative disease with complete response (CR) rates higher than 80% and long-term survival accounting more than 70% of patients receving R-2CdA. In the last recent years, the application of the deep sequencing thechniques allowed to detect the BRAFV600E mutation in up to 100% of the HCL patients; this mutation is also useful for the differentiating HCL cases from other indolent lymphomas, including the splenic marginal-zone or unclassifiable splenic lymphomas or leukemias, that are BRAF unmutated. Different authors described several available molecular techniques for detection of the BRAF mutation: a) the Sanger sequencing, with a sensitivity of 1-5% (Arcaini L); b) the pyrosequencing, which has a sensitivity of 5% (Laurini JA, Verma S); c) a specific quantitative real-time PCR, with a sensitivity of 2x10-4 (Schnittger S, Tiacci E). In this study, we assessed 20 untreated cases with splenic marginal lymphoma and further 20 HCL patients by a specific real-time PCR for the BRAFV600E mutation (qBiomarker Somatic Mutation PCR assays BRAF_476, SABiosciences), and by a newer PCR technique: the droplet PCR (PrimePCR™ ddPCR™ Mutation Assay). Our HCL patients received R-2CdA, achieved 75% of CR, being the CR the best response for 83% of patients. The 5-year overall survival was 87%, and PFS 66%. All patients, except one HCL case, showed the IgH clonality; as reported even by our group, the sensitivity of this method ranges from 1x10-3 to 1x10-4. When the same samples were tested for the BRAF mutation by the real-time PCR, all marginal cases resulted wild-type, whereas the

mutation was detected in 15 cases (75%) of the HCL patients. The negative case by the IgH resulted mutated by the droplet PCR. Sensitivity tests have been performed for both techniques, diluting a mutated DNA with a pool of wild-type DNAs, from 1x10 to 5x10-4. The sensitivity of the real-time PCR was 1x10-4, whereas that of the droplet PCR was 5x10-5, thus proving that the droplet PCR has got a sensitivity higher than half log. Moreover, the real advantage of the droplet PCR was the possibility of the absolute quantitation of the mutated alleles without necessity of a reference curve. Twelve HCL cases have been then monitored during the follow-up. At the end of therapy, the droplet PCR showed that the molecular tumor burden reduced of about 2 logs in the whole series; in all cases achieving the CR, the mutation burden reduced, both by real-time and by droplet PCR; in 2 relapsed patients, the RAF mutated allele burden increased; nevertheless, we were not able to predict the relapses, because samples were harvested in concomitance of relapse. In conclusion, we demonstrated that the droplet PCR could represent a valid tool either for differential diagnosis of HCL, or for monitoring HCL patients during treatment, with costs comparable to those of the real-time PCR.

PO-019

COPY NUMBER GAINS OF CHROMOSOME 1P36 LEAD TO PRDM16 OVEREXPRESSION IN AML PATIENTS

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Introduction. PRDM16 gene (1p36) is rearranged in AML/MDS with t(1;3)(p36;q21), t(1;21)(p36;q22) and t(1;12)(p36;p13). These translocations resulted in PRDM16 overexpression through juxtaposition to the enhancer of RPN1 at 3q21 or through fusion transcript formation with RUNX1 at 21q22 or ETV6 at 12p13, respectively. AML/MDS with t(1;3)(p36;q21) showed similar clinical and prognostic characteristics with AML/MDS with inv(3)/t(3;3) and EVI1 rearrangements. PRDM16 overexpression has been reported in AML without 1p36 rearrangements, but the mechanisms are still unknown and the studies are not conclusive. The aim of this study is to investigate PRDM16 involvement in cases with 1p36 abnormalities and to assess PRDM16 expression in a cohort of AML without 1p36 involvement. *Methods.* The study group was composed of 16 AML/MDS cases with 1p36 abnormalities and 80 AML without 1p36 involvement by conventional cytogenetic (CC). Cases were analysed by relative RQ-PCR and FISH using 3 BACs probes for PRDM16 and its flanking region. *Results.* We identified 16 cases with 1p36 abnormalities: FISH analysis of 15 available samples identified 5 cases with PRDM16 rearrangement. Three cases showed a t(1;3)(p36;q21), one a t(1;21)(p36;q22) and the last one an add(1)(p36) in CC. In 4 cases the breakpoint was at 5' of PRDM16, whereas in t(1;21) it was at 3' of PRDM16, which is a rare event. We were not able to identify the chromosome partner involved in the case with add(1)(p36). Other 3 cases showed copy number gains of PRDM16 identified as the presence from 3 to 5 signals with all the three probes used in interphase FISH. Metaphases FISH localized the site of amplification on the der(1)(p36) in two cases, whereas it was localized on unidentified chromosome in the last one. Rearrangements and amplification of PRDM16 were associated with overexpression by RQ-PCR. High levels of PRDM16 expression were observed in a significant subset of AML with normal karyotype (AML- NK) (12/25;48%) and with adverse cytogenetic prognostic group (3/11;27.3%) but they were also associated with isolated rare translocations (4/10;40%). In 3 cases with a complex karyotype FISH analysis detected an extra copy of PRDM16. *Conclusions.* PRDM16 gene is a frequent target of 1p36 abnormalities in AML. Copy number gains of PRDM16 are recurrent genetic abnormalities in AML with 1p36 abnormalities and, although less common, in AML with complex karyotype but undetectable 1p36 abnormalities. Copy number gains of chromosome 1p36 have not been previously associated with PRDM16 overexpression in patients with myeloid malignancies. We also demonstrated overexpression of PRDM16 in different subgroups of AML without PRDM16 rearrangements or amplification, especially in AML-NK subset. Since the poor prognosis associated to PRDM16, the role and prevalence of PRDM16 expression should be addressed further in a larger cohort of patient. Supported by University of Bologna RFO, BolognAil and Coop Reno.

PO-020

STAT5B GENE DYSREGULATION CORRELATES WITH LEF1 GENE EXPRESSION IN ADULT ACUTE PROMYELOCYTIC LEUKEMIA

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Introduction. Lymphoid enhancer-binding factor 1 (LEF1) is a downstream effector of the Wnt/ β-catenin signaling pathway, which controls cell growth and differentiation. Dysregulation of LEF1 expression may result in several disease patterns, as the Wnt signaling plays a pivotal role in development and cancerogenesis and also controls self-renewal, proliferation and differentiation of many types of stem cells. High LEF1 expression has been reported as a favorable prognostic marker in cytogenetically normal acute myeloid leukemia whereas it is associated with poor prognosis in adult B precursor acute lymphoblastic leukemia and in chronic lymphocytic leukemia. Moreover, marked downregulation of LEF1 is associated with disease progression in myelodysplastic syndromes. Recently, our group reported LEF1 expression as a prognostic factor in adult acute promyelocytic leukemia (APL). In hematologic malignancies has been reported a link between STATs transcription factor and LEF1; as STAT5b rarely is involved in molecular rearrangement with RARa gene in APL, we investigated whether there was a relationship between LEF1 and STAT5b gene expression. *Methods.* LEF1 and STAT5b expression was measured by real-time qPCR in 75 APL patients (median age 45 years, range 16 to 88 years). Advanced relative quantification analysis was performed using LightCycler 480 Software 1.5.1, based on the ΔΔCt method. LEF1 expression was measured using a RealTime intron-spanning ready assay recognizing all 4 major human LEF1 isoforms; STAT5b quantification was assessed by using specific primer selected according to Primer3 software. The β-glucuronidase (β-GUS) gene was employed as housekeeping gene and a pool of cDNA derived from BM cells of 5 healthy individuals was used as calibrator for normalization. APL samples were dichotomized at the median value and divided into two expression groups: low LEF1 (39 patients) with LEF1 values below the median value (LEF1low) and high LEF1 (36 patients) with LEF1 values above the median value (LEF1high). *Results.* Fifty-one (68%) APL patients showed a STAT5b expression that was higher than that observed in the healthy control group. Patients with LEF1high expression had higher amount of STAT5b transcript compared to that detected in the LEF1low patients group (2.2 *vs* 1.4 fold change; p=0.04). Moreover, there was a positive correlation between LEF1 and STAT5b gene expression ($r=0.61$, $p < 0.0001$); the relation between the two genes expression was more close in APL patients aged < 60 years compared to that observed in those with > 60 years ($r=0.64$, $p<0.0001$ *vs* $r=0.51$, $p=0.04$, respectively). *Conclusions.* Preliminary results from our study suggest that LEF1 gene expression in APL is linked to STAT5b gene dysregulation. As high LEF1 expression has recently been reported as a favorable prognostic marker in adult APL the identification of genes involved in LEF1 pathway plays a crucial role for clarifying molecular pathogenesis of APL.

PO-021

A NOVEL t(8;13)(q24.21;q14.3) REARRANGEMENT DEREGULATING MYC IN A B-CLL CASE WITH POOR OUTCOME

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Introduction. Chronic lymphocytic leukemia (CLL) is the most prevalent form of adult leukaemia in Western-world adults. The most frequent chromosomal abnormality is deletion of 13q, followed by deletions of 11q, trisomy 12, and deletions of 17p and 6q. These aberrations have been used as prognostic factors, defining a hierarchical risk model in B-

CLL patients, with del(17p) as the most aggressive one, followed by del(11q), del(6q), trisomy 12, normal karyotype, and del(13q) (the least aggressive). miR-15a and miR- 16-1, showing tumor-suppressing activity, are located within the minimum deleted region of 13q14.7, and exhibit significantly reduced expression in CLL. However, del(13)(q14) is associated with better survival and a favourable disease prognosis. *Methods.* We fully characterized a case of highly aggressive B-CLL showing a t(8;13)(q24.21;q14.3) translocation as the sole cytogenetic abnormality. FISH with BAC and fosmid probes, SNP array, long range PCR and Sanger sequencing analyses were performed to finely characterize the breakpoints on both derivative chromosomes 8 and 13. qPCR and immunohistochemistry assays were carried out to investigate the expression level of the genes involved in the rearrangement, at transcript and protein level, respectively. We also performed NOTCH1, IGHV, TP53, SF3B1, MYD88, FBXW7, and XPO1 mutation analyses by Next Generation Sequencing. *Results.* Both breakpoints of the novel t(8;13)(q24.21;q14.3) translocation were defined at nucleotide level resolution, detecting 2,6 Mb and 0,73 Mb deletions at the 13q14 and 8q14 breakpoint regions, respectively. Notably, both miR-15a and miR-16-1 were found as heterozygously deleted on der(13). Moreover, the breakpoint of the der(8) was mapped 7 Kb downstream the MYC locus. The evaluation of the MYC expression level in the present case *versus* a pool of B-CLL cases not carrying the same translocation disclosed an increase at both transcript [concerning one of its splicing variants (uc003ysh.1)], and protein level. In addition, we found a heterozygously mutated NOTCH1 (c.7541_7542delCT). *Conclusions.* We here describe an aggressive form of B-CLL showing a novel t(8;13)(q24.21;q14.3) translocation accompanied by the concurrent heterozygous loss of 13q (miR-15a and miR-16-1) and MYC deregulation due to a chromosomal position effect. Both deletions on der(8) and der(13), as well as the translocation breakpoints, were mapped at nucleotide level. Of interest, the MYC up-regulation here described is rarely observed in CLL and never associated with translocation involving 13q14. Although del(13q) is commonly reported in the least aggressive form of B-CLL, our patient showed an aggressive outcome of the disease, causing rapid death. The rapid progression of the disease in our patient thus confirmed NOTCH1 mutations as markers of poor prognosis for B-CLL, also when occurring together with the loss of miR-15a and miR-16-1.

PO-022

MIR-155 REGULATIVE NETWORK IN FLT3 MUTATED ACUTE MYELOID LEUKEMIA

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Among AML with normal kariotype (NK-AML) FLT3 internal tandem duplication (FLT3-ITD), is present in about 30% of patients, conferring unfavorable 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. 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-Transcription Factor (TF) connections by MIR@NT@N. Then we extracted, from the general network, the module of TF connected to miR-155. 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. Using a new cohort of newly diagnosed AML patients, we confirmed a strong up-regulation of miR-155 in the FLT3-ITD+ AML and we found a significant down-regulation of miR-155 target genes CEPB-beta and PU.1 and up-regulation of miR-155 regulator genes JUN and RUNX1. Our functional data showed that PKC412 related FLT3 inhibition, in MV4-11 FLT3 mutated cell line, causes down-regulation of miR-155 and increased level of mRNA and protein of miR-155 target PU.1 and downregulation of miR-155 regulator JUN. We obtained fitting results in experiment of miR-155 mimic in K562 cell line, in fact we showed a high increase of miR-155 post lentivirus transfection and an inverse correlation with the mRNA levels of its targets PU.1 (0,8 fold) and CEBPbeta (0,5 fold). Moreover silencing, by antagomiR lentivirus transfection, of miR-155 in primary AML blast cells causes up-regulation of its target PU.1 (2,5 fold) and CEBPbeta (3,8 fold). We described 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. 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), we thank NOVARTIS for providing us PKC412 molecule.

PO-023

UPREGULATION OF miR-29a IN NORMAL KARYOTYPE AML SHOWING DNMT3A MUTATION

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Acute myeloid leukaemia (AML) is a cytogenetically heterogeneous disorder with acquired recurrent chromosomal alterations detected in about 55% of adult patients; in the remaining 45% of cases of normal karyotype AML, a number of molecular abnormalities have been described involving several genes such as FLT3, NPM1, CEBPA, IDH1, IDH2 and DNMT3A. DNMT3A, a member of DNA methyltransferases, is mutated in approximately 22% of *de novo* AML patients with normal karyotype leading to adverse overall survival (OS), independently of age and the presence of FLT3 mutations. There are two major type of DNMT3A mutations. The first type is the highly recurrent set of mutations at codon R882 (R882-DNMT3A mutations); the second type is represented by all the other mutations in this gene (non-R882-DNMT3A mutations). Several studies have shown that genome-wide gene expression profiling can clearly distinguish the major cytogenetic groups, so providing a better understanding of the underlying disease biology. Our previous data demonstrated distinctive miRNA expression patterns in some genetic groups. To indagate about miRNA signature in NK-AML R882-DNMT3A mutated we performed quantitative real-time PCR (TaqMan Human MicroRNA Array, AB) , to study the expression of 384 known human miRNA in 7 selected de-novo AML cases showing DNMT3A mutations. We found, in almost all analyzed samples, a detectable amount of about 200 miRNA. We compare miRNA expression data with our previous results obtained in 31 AML DNMT3A unmutated and we focused on a strong up-regulation with a significant p-value of mir-155, miR-29a, miR-196b and miR-25. So we decide to investigate expression levels of these miRNAs in additional 10 new DNMT3A mutated AML patients and we confirm the up-regulation of 3 of them: miR-155 (6,56 fold p-value 0.022), miR-29a (fold 101 p-value < 0,001), miR-196b (44 fold p-value 0.002). The most interesting result is the high expression of miR-29a. miR-29a has been demonstrated to directly target 3'-UTR of DNMT3A resulting in a global hypomethylation but also miR-29s are able to directly suppress two major DNA demethylases, TET1 and TDG. These results suggest that miR-29a acts as an crucial regulator of DNA methylation probably protecting against changes in the existing DNA methylation status. The identification of the deregulation of miR-29a in presence of DNMT3A mutated gene may cause a perturbation of methylation status. The mechanisms through which DNMT3A mutations contribute to leukemogenesis are not yet characterized. Uncovering how DNMT3A mutations affect DNA methylation and epigenetic regulation of gene expression may have ramifications for treatment selection because DNA hypomethylating agents are increasingly used for up-front or salvage therapies in AML, and response to these drugs may be affected by alterations in DNMT3A function.

PO-024

FOXP1 AND TP63 INVOLVEMENT IN THE PROGRESSION OF MYELODYSPLASTIC SYNDROME WITH 5q- AND ADDITIONAL CYTOGENETIC ABNORMALITIES

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Introduction. The progression of low-risk del(5q) myelodysplastic syndrome (MDS) to acute myeloid leukemia (AML) is increased when associated with mutations of TP53, or with additional chromosomal abnormalities. However, to date the prognostic impact and molecular consequences of these rearrangements were poorly investigated. Single additional alterations to del(5q) by balanced chromosome rearrangements were rarely found in MDS. In particular, balanced alterations involving TP63 and FOXP1 genes were never reported in the literature. *Methods.* Here we report on a 79-year woman with an aggressive form of MDS with del(5q), no TP53 mutation (analyzed by exon-specific RT-PCR), and a novel complex rearrangement of chromosome 3 in bone marrow cells, fully investigated by FISH with BAC probes. FOXP1 and TP63 expression at protein level were evaluated by immunohistochemistry analysis using commercially available antibodies in both the patient under study as well as on four normal BM, additional MDS/AML cases with normal karyotype, or with 5q- as a sole cytogenetic abnormality, or with additional changes. *Results.* Our results revealed that the FOXP1 and TP63 genes were both relocated along chromosome 3 in the patient under study. Strikingly, immunohistochemistry analysis showed altered protein levels, disclosing that this rearrangement triggered the expression of FOXP1 and TP63 genes. We performed the same analysis on four normal bone marrow samples, as well as in additional MDS/AML cases with normal karyotypes, or with 5q- as a sole cytogenetic abnormality, or with additional changes. The overall results showed that TP63 was negatively expressed in all the control cases. Conversely, FOXP1 was negatively expressed only in normal bone marrow and one MDS case with a normal karyotype. The remaining cases showed a variable level of protein expression, directly proportional to the percentage of myeloid precursor cells found in each case. Of note, AML cases in our cohort displayed higher expression levels of FOXP1 than MDS. In particular, our patient showed the highest FOXP1 expression level among MDS cases, since she had a high percentage of positive myeloid precursors in her bone marrow. Moreover, we observed that the majority of the MDS/AML patients refractory to therapies, including our case, showed the highest FOXP1 expression levels. *Conclusions.* We here report a notable MDS case, which rapidly evolved to AML, harboring 5q- and dysregulation of both FOXP1 and TP63. We also document an apparent role of FOXP1 and TP63, so far poorly documented, in the progression of MDS in our patient who is lacking mutations in the TP53 tumor suppressor gene, normally associated with poor outcome in MDS with 5q-. Finally, we found FOXP1 as activated in other patients with MDS and AML, showing that it is an important, recurrent event. Thus, our results may suggest a possible broader role of FOXP1 in the pathogenesis and progression of MDS and AML.

PO-025

MOLECULAR CHARACTERIZATION OF FOUR MYELOID LEUKEMIA CASES WITH A t(15;21) TRANSLOCATION DISRUPTING RUNX1

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Introduction. RUNX1 (Runt-related transcription factor 1) gene located in chromosome 21q22, encodes a transcription factor crucial for normal hematopoiesis. RUNX1 is described as a target of chromosomal rearrangements, mainly translocations or deletions, in leukemia. Among translocations, more than 55 cytogenetic bands are described as partners of RUNX1, although only in 22 of them a partner gene has been identified. Some of the translocations are recurrent, such as t(8;21)(q22;q22) generating 5'RUNX1/3'CBFA2T1 in AML M2; t(16;21)(q24;q22) with 5'RUNX1/3'CBFA2T3 in AML-M1/M2 patients, and the t(3;21)(q26;q22), in both *de novo* and secondary AML, fusing RUNX1 to MDS1, RPL22L1 or EVI1. Recently, our research group identified two novel fusion genes of RUNX1, involving CBFA2T2 or C20orf112a in two cases with a t(20;21) translocation. *Methods.* Here we describe four cases (three AML and one CML) showing t(15;21) translocations involving RUNX1. Reiterative FISH experiments were performed to characterize the breakpoint regions on both derivative chromosomes 15 and 21. Appropriate clones (BAC or fosmids) were selected according to the GRCH 37/hg19 UCSC Human Genome Browser release. Moreover, RUNX1 expression level was evaluated by qPCR and, to detect putative chimeric transcripts, RT-PCR assays were performed. *Results*. FISH analyses revealed that the breakpoints within RUNX1 were located between exons 5-6 (CML case) and 6-7 (AML cases) of the longest gene transcript variant 1 (NM_001754.4). The mapped breakpoints on chromosome 15 did not show the same position, except for two cases showing the recurrent involvement of TCF12 (transcription factor 12) at 15q21.3. However, the juxtaposition of TCF12 to RUNX1, with an opposite transcriptional orientation, excluded the origin of a fusion transcript. Another breakpoint was mapped within SIN3A (SIN3 transcription regulator family member A) at 15q24.2, described as deregulated in many cancers. Here, the translocation juxtaposed the 5'end of SIN3A to the 3'end of RUNX1, with the same transcriptional orientation. However, RT-PCR experiments ruled out the occurrence of a SIN3A/RUNX1 fusion transcript. The breakpoint in the fourth case, located within band 15q26, is presently under definition by FISH analysis. qPCR experiments to assess the expression level of RUNX1, as well as further RT-PCR assays to identify aberrant transcripts generated by the translocations, are also in progress. *Conclusions*. We here report four myeloid leukemia cases harboring t(15;21) translocations involving RUNX1. Since no fusion gene was detected at this stage, our results suggest that the described translocations t(15;21) may have a leukemogenic role by truncating RUNX1, event already described by us and others in leukemias with RUNX1 rearrangements.

Chronic Lymphocytic Leukemia and Chronic Lymphoproliferative Disorders I

PO-026

MESENCHYMAL STROMAL CELLS IMPROVE SURVIVAL OF B-CLL CELLS: ROLE OF CELL-CELL CONTACT AND SOLUBLE FACTORS IN THE MICROENVIRONMENT

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Introduction. The malignant behavior of chronic lymphocytic leukemia (CLL) cells cannot be only ascribed to their intrinsic features, but also to factors originating from the surrounding microenvironment. At the active sites of disease, such as bone marrow (BM) and secondary lymphatic tissues, CLL B cells engage complex 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 and are involved in supporting leukemic B cell survival. The aim of this work was to evaluate how MSCs influence CLL B cell behavior and which soluble factors could be involved. *Methods*. MSCs isolated from the BM of 46 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 CLL-MSCs. Apoptosis was measured by Annexin V test and western blotting analysis. Chemokines and cytokines released in culture supernatants were collected for the human Bio-Plex™ 27 plex Cytokine Assay. *Results*. In presence of CLL-MSCs, we observed an extended survival of leukemic cells (60%±17.3 with MSCs *vs* 14%±11.7 with medium alone). Through a transwell system to avoid lymphocytes-MSCs direct contact, we observed that CLL B cell survival in presence of MSCs was minimally reduced (60%±17.3 with MSCs *vs* 52.4%±23.5 with MSCs in transwell system), suggesting that the anti-apoptotic effect is mainly ascribed to soluble factors produced by MSCs. In order to identify soluble factors responsible for the MSCs anti-apoptotic effect, CLL-MSCs cytokine/chemokine secretion profile was evaluated before and after leukemic B cells exposure. We observed a stronger increase of IL-8, IL-15, CCL11 and CXCL10 production under co-culture conditions, suggesting their potential involvement in leukemic B cell survival. Considering the high heterogeneity in CLL B cell viability in response to CLL-MSC pro-survival stimuli, we evaluated the caspase dependent cleavage of PARP on 24 CLL samples after 7 days co-culture with CLL-MSCs. The analysis of this protein after CLL-MSCs exposure, led us to subdivide CLL samples into two groups: clones which depend (dependent) and clones which, at least in part, do not depend (independent) on the microenvironment signals for their survival. *Conclusions*. We demonstrate that CLL-MSCs co-culture represents a reproducible *in vitro* system mimicking the *in vivo* bone marrow conditions, pointing out that the heterogeneity of the disease is reflected also by CLL B cell capability to respond to favorable signals from CLL-MSCs. Since patients carrying a leukemic clone strictly related to environmental stimuli could be positively affected by the action of drugs targeting the cross-talk with marrow microenvironment, we are now in the process of determining CLL-niche interactions involved in CLL B cell survival.

PO-027

BCR SIGNALLING TUNING IN CLL: ROLE OF c-CBL AND CIN85

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Introduction. In B cell chronic lymphocytic leukemia (CLL) little is known about the alterations affecting the mechanisms involved in the preservation of homeostasis of Signal Transduction Pathways (STPs). Homeostasis of STPs is maintained by different molecules. c-Cbl (c-Casitas B-lineage lymphoma), an E3 ubiquitin ligase and adapter molecule, and CIN85 (Cbl-interacting protein of 85kDa), an adapter proteins that function as docking partners for several signaling proteins, control protein kinase degradation and receptor down-regulation. The accumulation of clonal B lymphocytes in CLL is mostly due to apoptosis resistance but also to proliferative activity. Abnormalities of molecules involved in STPs are connected to CLL pathogenesis and a critical role has already been ascribed to B-cell receptor (BCR)-Lyn axis. We reported that Lyn kinase, is 2.5- up to 5-fold over-expressed in leukemic respect to normal B cells. The evidence that Lyn mRNA level was similar in normal and neoplastic B cells suggested that the anomalous Lyn protein expression was not related to differences in gene transcription and/or mRNA stability but to a deregulation in Lyn turnover. For this reason, we investigated c-Cbl and CIN85, that in normal B cells are involved in the ubiquitin-dependent Lyn degration and in the down-regulation of BCR signaling. *Methods.* To characterize CIN85 and c-Cbl at basal conditions in leukemic B cells isolated from 30 patients we analyzed the protein level by western blotting analysis. To evaluate the interaction between CIN85, c-Cbl and Lyn in CLL B cells we performed a co-immunoprecipitation assay followed by western blotting analysis. *Results*. We demonstrated that both CIN85 and c-Cbl expression detected in CLL B lymphocytes was 1.5 to 2-fold higher with respect to normal B cells ($p<0.001$). We also found that Lyn in neoplastic B cells did not co-immunoprecipitate with CIN85 and c-Cbl at steady state conditions as reported for normal B lymphocytes, probably due to Lyn association with Hsp90. *Conclusions*. These preliminary results prompt us to investigate the role of CIN85 and c-Cbl in the development of neoplastic clone. Considering that Lyn is over-expressed in CLL B cells, the evidence that these two regulator molecules are over-expressed, but not associated with Lyn, suggest that in CLL they could not play as negative regulators of Lyn expression and BCR signalling, as happens in normal B cells.

PO-028

PROGNOSTIC ROLE OF CpG CYTOGENETIC IN LOW-RISK RELAPSED CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS

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Introduction. Chronic lymphocytic leukemia (CLL) exhibits a remarkable clinical heterogeneity that likely reflects the underlying biological and genetic diversity. In CLL, recurrent chromosomal abnormalities, detected by fluorescent *in situ* hybridization (FISH), have been reported and stratified by the model proposed by Dohner *et al.* in 2000. However, FISH, focusing on specific genetic lesions, may fail to detect additional chromosome abnormalities (ACA) or complex karyotype (CK), which are detected by cytogenetic analysis only. The aim of this study was to investigate the role of stimulated cytogenetic analysis on the outcome of low-risk relapsed CLL. *Methods*. In this study we have investigated 97 relapsed CLL patients with isolated 13q- or normal FISH; 89 referred to the Hematology and Clinical Immunology Unit of Padua, 6 to Hematology Unit of Rovigo, and 2 to Oncology Unit of Vittorio Veneto. For all these patients, stimulated cytogenetic analyses were performed at relapse after the first-line of therapy. Karyotype was obtained after 72 hours of stimulation with 500μM CpG oligonucleotide and 20 U/ml of IL-2. Categorical variables were analyzed with Fisher exact test. Overall survival (OS) was defined as time from relapse to death (event) or last know follow-up (censored). Survival curves were estimated using the Kaplan-Meier method. Differences in survival distribution were analyzed by Log-rank tests. All analyses were performed at a significance level of 5%. *Results*. By FISH analyses performed on our cohort of patients, 72% showed 13q- and 28%

had a normal FISH. 56 patients were male and the median age at relapse was 69 years. ACA and CK, this latter defined by the presence of 3 or more chromosomal lesions, were found in 31% (n=30) and 13% of subjects (n=13), respectively. When we considered only patients with ACA the prevalence of CK raised to 43%. In 6 cases ACA involved immunoglobulin locus. ACA and CK were independent from IGHV mutational status, CD38 and ZAP70 expression. Subsequently, we analyzed the impact of stimulated cytogenetic analyses on CLL patients' outcome demonstrating that ACA and CK were associated with shorter OS (Log-rank test $p<0.0080$ and $p<0.0024$, respectively). Moreover, the 2-years OS were 61% and 89% for patients with and without CK, respectively. Among the ACA group we also observed that subject with CK had an adverse prognosis than patients without CK (median OS was 20.4 months *vs* not reached, Log-rank test $p<0.0322$). These data point that a CK rather than the presence of ACA per se negatively impacts on patients' survival. *Conclusions*. CpG cytogenetic analysis demonstrated that ACA and CK were present in a remarkable amount of low-risk CLL patients at relapse. Acquisition of ACA or CK at relapse is an independent adverse prognostic factor, even among patients with 13q deletion of normal FISH. Our study supports the usefulness of stimulated cytogenetic analyses to accurately define the prognosis of patients with CLL.

PO-029

DEATH RECEPTOR 3 EXPRESSION AND SERUM LEVEL OF SOLUBLE TL1A CORRELATE WITH INDOLENT, EARLY STAGE B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction. B-cell chronic lymphocytic leukemia (B-CLL) accounts for approximately 30% of leukemia diagnosed in the Western countries and shows an increasing incidence with the age of the population. Analysis of survival times has led to the establishment of staging systems according to various prognostic markers, including Rai stage, IGHV mutational status, expression of CD38 and Zap70. Clinically, B-CLL is a heterogeneous disease with variable presentation and evolution. Two major subtypes can be distinguished, indolent and aggressive, which require different treatment strategies. However, prediction of the clinical course of individual patients with the same stage and risk group remains variable. Receptors of the TNFR superfamily play a fundamental role in promoting the growth of B-cell chronic lymphocytic leukemia. Death receptor (DR) 3 is a TNFR-superfamily member expressed in lymphocyte-enriched tissues. DR3 and its ligand, TNF-like ligand 1A (TL1A), are implicated in regulatory mechanisms of adaptive immune response under physiological and pathological settings. Recently, we have demonstrated that DR3 is expressed on the surface of B cell receptor (BCR)-stimulated B cells and interaction of DR3 with TL1A reduces proliferation of suboptimally activated healthy B cells *in vitro*, without affecting cell survival. These findings prompted us to examine the expression of DR3 and TL1A in B-CLL and their possible role as risk factors for disease progression. *Methods.* DR3 surface expression of 37 B-CLL samples was measured by flow cytometry at baseline and following stimulation with F(ab')2 anti-human IgM conjugated to latex microspheres. TL1A serum levels of 26 B-CLL samples were measured by ELISA. Correlation analysis with clinical and biological parameters were performed using GraphPad Prism software. *Results.* Here, our preliminary results show that DR3 is expressed on the surface of activated CLL B cells and TL1A is present in the serum of B-CLL patients. Moreover, we show that BCR-induced DR3 expression is more frequently detected in samples with indolent, early-stage disease (Rai 0). The relevance of these findings has been confirmed by serum TL1A measurement showing that higher serum levels of TL1A are more frequently detected in B-CLL patients with favorable prognostic parameters (*i.e.* absence of CD38 expression) and early-stage disease. *Conclusions.* Taken together, these findings suggest that in B-CLL the TL1A/DR3 modulatory function on cell metabolism, in the presence of antigen stimulation, is a feature of indolent, early-stage disease. Thus, we can assume that these factors could be useful in monitoring disease activity and may be of prognostic relevance in B-CLL.

PO-030

VISCERAL LEISHMANIASIS AS LYMPHOPROLIFERATIVE DISORDERS COPYCAT

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Introduction. Visceral leishmaniasis (kala azar) usually occurs with fever, splenomegaly and blood exams abnormalities, such as cytopenias and hypergammaglobulinemia. Early infection typically presents with skin lesions, such as nodules or plaques. *Methods*. We report on 6 either immunocompetent or immunocompromised patients, admitted between September 2012 and January 2014, because of either cytopenias or fever or splenomegaly with or without adenopathies. All of them underwent bone marrow (BM) aspirate and/or trephine biopsy for morphologic and flow cytometry examinations, together with molecular analyses for lymphocyte clonality and Leishmania spp detection by nested polymerase chain reaction (nPCR). *Results*. All patients (1 female and 5 males, age range 45-84 years) were living in the neighborhood of Modena and Bologna (Italy). Four of them had no previous medical history, while an elderly male patient suffered from B-chronic lymphocytic leukemia (CLL) and the woman was HIV-seropositive. Clinical presentation was heterogeneous: all patients presented with at least one cytopenia, usually leukopenia with mild to severe neutropenia and thrombocytopenia; fever occurred in 5 cases; three of them showed splenomegaly and adenopathies; one presented with acute liver failure; severe polyclonal hypergammaglobulinemia was recorded only in the patient in follow-up for CLL. BM smear cytological evaluation on panoptic staining led to recognition of Leishmania amastigotes, either into macrophage cytoplasms or dispersed, in 3 out of 6 cases (Figure 1). nPCR for Leishmania spp, carried out on BM samples, was positive in all cases, allowing a diagnosis of visceral leishmaniasis also in 3 three patients in which Leishmania amastigotes were not found by microscopic observation. Immediately after diagnosis, targeted therapy with liposomal B amphotericine (3 mg/Kg/daily for 5 days) was started, with hematologic recovery and clinical improvement observed in all patients by one month. *Conclusions*. Our small series suggests the importance to consider Leishmania spp protozoal infection in the differential diagnosis in patients presenting with at least one of the following symptoms: fever, one or more cytopenias, inhomogeneous splenomegaly and/or lymph node enlargement. A peculiarity of our cases, diagnosed with visceral leishmaniasis, clinically mimicking aggressive lymphoproliferative disorders, in a non-endemic area, relies on the heterogeneous clinical manifestations, with disseminated infection in the absence of skin involvement and usually without hypergammaglobulinemia. We would like to remark the relevance of collecting BM aspirate samples not only for morphologic examination, but also for molecular analysis, which is currently the highest sensitive diagnostic method for leishmaniasis. Prompt start of specific therapy against Leishmania is essential to obtain resolution of symptoms and parasitic eradication.

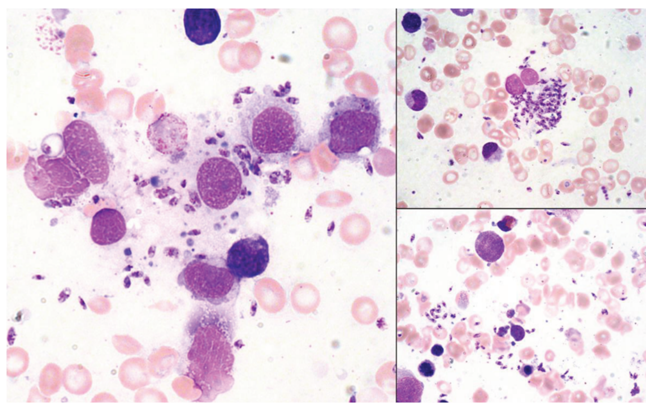

Figure 1.

PO-031

EXTERNAL AND MULTICENTRIC VALIDATION OF THE MD ANDERSON CANCER CENTER NOMOGRAM AND PROGNOSTIC INDEX FOR CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) PATIENTS: ANALYSIS OF 1502 CASES

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Introduction. In 2007 Wierda *et al.* proposed a predictive nomogram and a simplified prognostic index built on basic clinical characteristics (age, sex, Rai stage, number of lymph node groups) and laboratory parameters [β2-microglobulin (β2M) and absolute lymphocyte count (ALC)] universally applicable to previously untreated CLL patients to predict survival. We performed a validation of the nomogram and the index in an independent series of Italian patients. *Methods.* Databases of 4 Italian centers including roughly 3000 CLL patients diagnosed between 1980 and 2013 were used to evaluate the validity and reproducibility of the MDACC nomogram and prognostic index. Data regarding all six parameters were available for 1502 cases. Nomogram and prognostic index score were calculated using the method proposed by Wierda *et al*. Progression free survival (PFS) and overall survival (OS) analyses were performed using the Kaplan-Meier method. The prognostic impact for the outcome variable was investigated by univariate and multiple Cox regression analysis. *Results*. The median age of the 1502 patients was 67 years (range 27-94) with 55.7% male. The majority of patients had Binet stage A (82.9%), 847 cases (56.4%) had Rai stage 0; moreover 151 cases (10.1%) satisfied the 2008 NCI/WG criteria for clinical monoclonal B-cell lymphocytosis (cMBL) (<5.0x109 B lymphocytes/L in the peripheral blood and no apparent lymph node, spleen or liver enlargement). All 6 parameters involved in the prognostic index were found to be independently associated with survival in this analysis (age: HR 1.085, 95%CI 1.071-1.1, P<0.0001; sex: HR 1.548 95%CI 1.204-1.989, P=0.001; ALC: HR 1.006, 95%CI 1.003-1.01, P<0.0001; number of lymph node groups: HR 2.222, 95%CI 1.671-2.956, P<0.0001; β2M: HR 1.216 95%CI 1.16-1.275, P<0.0001). The median nomogram score was 87 (range 27.4-181.8). The nomogram resulted accurate in predicting survival (Harrell c-index=0.82). According to the prognostic index 38.7% of patients were classified as low-, 58.3% as intermediate- and 3% as high-risk. The estimated median survival times were: not reached for low-risk, 13.4 years for intermediate-risk, and 3.4 years for high-risk. The estimated median and 5- and 10-year survival by prognostic index risk category were similar to those originally reported. The prognostic index remained significantly associated with OS also when patients were sub-grouped by period of diagnosis (1980-1995, P<0.0001; 1996-2004, P<0.0001; 2005-2013, P<0.0001). The prognostic index risk category remained a predictor of survival when analysis was limited to Rai stage 0 (P<0.0001) and cMBLs (P=0.009). Finally the prognostic index also allowed prediction of PFS in all 1502 patients (P<0.0001), in Rai 0 cases (P<0.0001) and in cMBLs (P=0.035). *Conclusions*. Our results confirm the ability of the nomogram and the prognostic index to predict prognosis, even in early stage disease cases. The study also extended the utility of the index to cMBLs.

PO-032

THE ASSOCIATION OF DEXAMETHASONE+SIMVASTATIN TO OVERCOME ENVIRONMENT-MEDIATED CHEMORESISTANCE OF 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 promotes survival and *in vivo* accumulation of tumor cells and confer a multidrug resistance phenotype to CLL B cells. The inhibition with simvastatin (SIM) abrogated the mevalonate (Mev) pathway-dependent Ras/ERK1-2 and RhoA/RhoA kinase signaling cascades, the HIF-1α/P-glycoprotein axis and reversed the constitutive and stromal cell-induced resistance of UM cells to doxorubicin. Statins induce apoptosis of CLL and lymphoma cells *in vitro* and increase the susceptibility of CLL and lymphoma cells to dexamethasone (Dex). The resistance to Dex was associated to an increased Pparα expression and activity. The aim of this study was to determine *in vitro* the ability of SIM in association with Dex to induce CLL cell death, to determine the lowest effective concentration of drugs to translate in a clinical trial and to better understand the biochemical mechanism of drug resistance and the metabolic differences between M and UM CLL cells. *Methods.* M and UM CLL cells were cultured in the presence and in the absence of murine stromal cells (M2-10B4) and exposed to low doses of SIM (from 10 nM to 100 nM) and Dex (from 10 nM to 500 nM) alone or in combination. The cholesterol and ubiquinone activity was measured by metabolic radiolabelling with [14C]-mevalonic acid and thin layer chromatography; electron flux through mitochondrial chain by spectrophotometric assay; ATP synthesis by luciferin/luciferase-based assay, to measure the changes in aerobic energy metabolism. Mitochondrial membrane potential was measured by JC staining, to assess eventual mitochondrial damages. Fatty acids beta-oxidation was measured by metabolic radiolabelling to measure crucial pathways in aerobic energy metabolism. Apoptotic cell death was detected by flow cytometry using Annexin V and propidium iodide. *Results.* UM CLL cells were significantly more sensitive to Dex than M CLL cells also at low doses. UM CLL cells treated with the association Dex+SIM have a reduced viability and a lower activity of Mev pathway than UM CLL cells treated with Dex or SIM alone. Dex induced an increased fatty acids beta-oxidation and a higher mitochondrial membrane potential and ATP synthesis in UM CLL cells, but not in M. In the presence of Dex+SIM this effect was abrogated. When M and UM CLL cells are cultured with Dex or SIM alone, M210B4 protect their viability. In M cells, M210B4 protect the viability of cells also when Dex is associated with SIM, instead in UM cells the association between the two drugs counteract the protection of M210B4. *Conclusions.* These data indicate that the combined action of Dex+SIM could be a novel potential strategy to circumvent basal and environment-mediated chemoresistance of CLL cells.

PO-033

NOVEL GENETIC MARKERS MAY SURROGATE FOR IGHV MUTATIONAL STATUS IN EARLY STAGE CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) IN PREDICTING DISEASE PROGRESSION

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Introduction. Considerable research has focused on the identification of aggressive disease markers in CLL. The most popular include CD38, IGHV mutational status and its surrogate marker ZAP-70, which has been used to identify patient groups with divergent clinical courses.

Although first identified in gene expression profiling studies (GEP) in patients with CLL, the evaluation of ZAP-70 expression is currently based on flow-cytometry which often produces inconsistent results due to lack of standardized protocols. We investigated novel gene markers which may surrogate IGHV mutational status. *Methods.* We performed an independent supervised analysis by IGHV mutational status in CLL-Training (102 cases) and CLL-Validation (114 cases) staking into consideration all those genes showing a fold change superior or equal to that of ZAP-70. RNA obtained from highly-purified B-cells was employed for both IGVH mutation analysis and GEP profiling, using a high resolution microarray (GeneChip®Gene1.0 STArray, Affymetrix Inc). *Results.* Thirty-one genes (23 up-and 8 down-regulated) and 23 genes (18 up-and 5 down-regulated) showed a fold change superior or equal to that of ZAP-70 in CLL-Training and CLL-Validation sets, respectively; notably, 20 common genes (15 up and 5 down) overall were found to be differentially regulated in the 2 cohorts analysed. Two (SNORA70F and NRIP1) of the 5 down-modulated and 6 (SEPT10, ZNF667, TGFBR3, MBOAT1, LPL and CRY1) of the 15 up-modulated genes were found to be significantly associated to a reduced risk of disease progression in both training and validation sets. Notably, when all of the above mentioned genes were forced in a Cox multivariate model together with IGHV mutational status, only CRY1 (H.R. 2.3, 95% C.I. 1.1-4.9, P=.027) and MOABT1 (H.R. 2.1, 95% C.I. 1.1-3.7, P=.018) maintained their independent prognostic impact, while IGHV (H.R. 1.4, 95% C.I. .7-2.7, P=.4) and SNORA70F (H.R. 0.6 95% C.I. 0.3-1.2, P=.14) lost their prognostic power, supporting the hypothesis that these two genes could surrogate the predictive value of IGHV mutational status. The two discriminating genes, MBOAT1 and CRY1 were further validated using quantitative real-time PCR. All the analyses confirmed the microarray results. Pearson correlation coefficients of the expression of each transcript as determined by microarray or Q-RT-PCR were 0.90 and 0.78, respectively. *Conclusions.* Given the pressing need of novel special strategies to surrogate IGHV gene mutation status for determination of prognosis, we identified definite transcriptional models that are associated with IGHV gene mutations that may have biological and clinical relevance in IGHV distinct CLL groups. Our data suggest that CRY1 and MBOAT1 may represent novel gene markers able to surrogate the predictive power of IGHV mutational status.

PO-034

PHOSPHORYLATION OF PP2A BY LYN IS A KEY PHENOMENON IN THE CANCER PHENOTYPE OF B-CLL

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Introduction. B-Chronic Lymphocytic Leukemia (B-CLL) is characterized by clonal expansion with accumulation of mature CD5+ B lymphocytes in the peripheral blood, bone marrow and secondary lymphoid organs. The B-cell receptor in B-CLL cells exhibits tonic signaling mediated by a variety of kinases such as Btk, PI3Kdelta, Syk, and Lyn. Amongst these, Lyn, a Src family kinase (SFK), is overexpressed and is delocalized in the cytosol within an aberrant multiprotein complex stabilized by Hsp90,(1) accounting for the constitutive activity of Lyn itself and the high level of tyrosine phosphorylation, which in turn leads to resistance to apoptosis.(2) Among the potential substrates of Lyn within the phosphoproteome of B-CLL cells with a role in supporting leukemic cells survival, we have recently identified procaspase-8, whose phosphorylation at Tyr380 by Lyn promotes the formation of an inactive homodimer, thereby inhibiting caspase-dependent apoptosis.(3) The aim of this work was to identify novel cytosolic molecules acting as anti-apoptotic players in B-CLL cells owing to its phosphorylation by the constitutively active and delocalized pool of Lyn. *Methods*. We analysed B lymphocytes from normal donors and CD5+/CD19+ B-cells purified from 40 CLL patients. *Results*. By biochemical methods and a bioinformatics approach, we established that in B-CLL cells Lyn phosphorylates the catalytic subunit of the protein phosphatase 2A (PP2A) at Tyr307, resulting in its inactivation. This is in agreement with a role for SFKs as upstream negative regulators of PP2A,(4) Moreover, PP2A phosphorylation caused a stronger interaction with its cellular inhibitor SET, which is known to be overexpressed in B-CLL,(5) which further inhibited PP2A activity. As a result, downstream PP2A targets, AKT, GSK-3β and SHP-1 remained constitutively activated exterted their pro-survival action. Experiments conducted in the presence of PP2A inhibitors (okadaic acid) and activators (FTY720 and OPN449) SFK inhibitors (dasatinib, PP2 or saracatinib), HSP90 inhibitors (which can disrupt the Lyn aberrant cytosolic complex), all confirmed that inhibition of PP2A contributes to the cancer phenotype of B-CLL cells. *Conclusions*. Our findings show that PP2A is a crucial regulator in leukemogenesis and the survival of B-CLL cells, therefore emerging as a potential therapeutic target in CLL. Further efforts are necessary to elucidate the mechanism of SET interaction with PP2A in CLL cells and to clarify the mechanism of action of PP2A activators supporting the rational development of new therapies. Moreover, we demonstrated once more how Lyn abnormal state is an essential feature in the pathogenesis of CLL and should be considered as a central player in this disease.

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PO-035

MICROENVIRONMENTAL INPUTS MEDIATED BY B-CELL RECEPTOR (BCR) STIMULATES NOTCH1 ACTIVITY IN CHRONIC LYMPOCYTIC LEUKEMIA (CLL) CELLS HARBORING NOTCH1 MUTATIONS

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CLL is characterized by the accumulation of mature B cells in the peripheral blood (PB) and lymphoid organs. The clinical course is highly heterogeneous in terms of progression, therapeutic response and outcome. Identification of prognostic markers for CLL patients has therefore been a priority in the research agenda. Next generation sequencing has been used to reveal previously unrecognized genetic lesions and identify potential novel markers. Among them, NOTCH1 mutations are the most frequent somatic aberration in CLL, found in 5-10% at diagnosis, with frequency increasing to ~20% in progressive/relapsed cases. NOTCH1 mutations identify patients with shorter overall survival, time to treatment and progression free survival, and represent an independent prognostic factor for the disease. Most mutations occur in exon 34, leading to PEST domain loss, resulting in NOTCH1 impaired degradation, stabilization of the intracellular domain (NICD) and deregulated signaling, both canonical and non-canonical. Microenvironment interactions are critical to initiate and maintain signaling, even in the presence of mutations, otherwise NOTCH1 activity is rapidly switched off. This is in line with the view of CLL as a compartmentalized disease, with a quiescent fraction in PB and a proliferating tumor load in the lymph nodes and bone marrow. Here, microenvironment-driven pathways, such as the BCR pathway, provide co-stimulatory signals that promote CLL cells proliferation and survival, likely leading to accumulation of genetic lesions or expansion of more aggressive sub-clones. The aim of the study was to explore the cross-talk between the NOTCH1 and the BCR pathways and whether NOTCH1 mutations may play a role in this context. Stimulation with IgM resulted in an effective BCR signaling, independently of NOTCH1 mutational status, as witnessed by the rescue from spontaneous apoptosis and increased expression levels of CCL3, a validated target gene of the BCR pathway. However, at a variance with NOTCH1 WT cases, in NOTCH1 M patients BCR activation also increased NOTCH1 expression and activity. Of note, non-canonical pathway, as evaluated by expression of DTX1, was selectively interested. This is in line with previously pub-

lished data, showing that culture of NOTCH1 M CLL cells in conditions that mimic a lymphoid microenvironment *in vitro*, selectively activates non-canonical NOTCH1 pathway, and protects cells from drug-induced apoptosis. Finally, preliminary data revealed a more sustained response to ibrutinib in NOTCH1 WT patients than in M, suggesting a role for NOTCH1 mutations in restraining ibrutinib activity, and paving the way to combine NOTCH1 and BCR inhibitors as a therapeutic strategy to selectively target NOTCH1 M CLL patients.

PO-036

EXTRACELLULAR NICOTINAMIDE PHOSPHORIBOSYLTRANSFERASE (NAMPT) SHAPES THE CLL MICROENVIRONMENT PROMOTING MACROPHAGE M2 POLARIZATION VIA A NON-ENZYMATIC MECHANISM

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The cancer-associated microenvironment provides malignant cells with a cocktail of signals that increase survival, reprogram their metabolism and help escape from the action of the immune system. Nicotinamide phosphoribosyltransferase (iNAMPT) is the rate-limiting enzyme in NAD biosynthesis. An extracellular form of this protein (eNAMPT) exerts cytokine/adipokine-like actions. Both iNAMPT and eNAMPT levels are increased in tumors, suggesting that this molecule is at the crossroad between metabolism and inflammation. Here we show that NAMPT mRNA, as well as intracellular and plasma protein levels are significantly up-regulated in chronic lymphocytic leukemia (CLL) patients compared to healthy donors. eNAMPT, secreted by activated CLL lymphocytes in the extracellular milieu, polarizes resting monocytes towards macrophages with an M2 phenotype. These cells express high levels of CD163, CD206 and indoleamine 2,3-dioxygenase (IDO) and secrete immunosuppressive cytokines, such as IL-10, CCL18 and IL-6. Furthermore, NAMPT-primed M2 macrophages reduce antigen-driven T cell proliferation and support Treg expansion. Once exposed to eNAMPT, M2 macrophages activate a signaling pathway characterized by ERK1/2 phosphorylation and by the activation of STAT3 and NF-kB. These effects are independent of the enzymatic activity, as inferred by the use of an enzymatically-deficient NAMPT mutant. Overall, these results reveal that eNAMPT is a critical element in the induction of an immunosuppressive and tumor-promoting microenvironment in CLL, in a way apparently independent of its enzymatic activities.

PO-037

PROGNOSTIC ROLE OF ZAP-70 IN EARLY STAGE CHRONIC LYMPHOCYTIC LEUKEMIA (CLL): RESULTS OF THE ANALYSIS FROM A PROSPECTIVE MULTICENTER OBSERVATIONAL STUDY USING DIFFERENT FLOW-CYTOMETRY (FC) METHODS

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Introduction. The evaluation of ZAP-70 is often inconsistent due to lack of standardization consensus. We investigated i) the most accurate methods of identifying ZAP-70 expression levels; ii) the predictive power of ZAP-70 expression for progression free survival (PFS). *Methods.* In a prospective cohort of 487 newly diagnosed Binet stage A CLL (clinicaltrial.gov ID: NCT00917540) ZAP-70 expression was evaluated by three FC *Methods.* using either an isotype-matched antibody as negative control (ZAP-70 I/C), the expression of ZAP-70 in normal T cells as an internal positive control (ZAP-70 T), or the ZAP-70 MFI ratio in T/B-cells (ZAP-70 T/B). *Results.* Cases were randomly split into CLL-Training and CLL-Validation cohorts. ROC analysis disclosed that 40% was the most suitable cut-off value to distinguish those patients with IGVH mutated from IGVH unmutated for ZAP-70 I/C and ZAP-70 T, while 1.5 represented the best threshold ratio for ZAP-70 T/B. However, we also devised cases according to other thresholds (*i.e.*, 20% and 30% for ZAP-70 I/C and ZAP-70 T, as well as 2 and 2.5 for ZAP-70 T/B). Cox analyses of the three different ZAP-70 detection methods for both training and validation sets was performed. For the training set, for ZAP-70 I/C only the threshold value of 20% was not significant, ZAP-70 T threshold was significant at 40%, while cut-offs of ZAP-70 T/B ≥1.5 and ZAP-70 T/B >2 could best discriminate clinical outcome. In the validation set, ZAP-70 I/C 30% was no longer significant, while ZAP-70 I/C 40% and 40% ZAP-70 T remained significant, and a ZAP-70 T/B ≥1.5 retained significance for both methods. Therefore, ZAP-70 I/C 40%, ZAP-70 T 40% and ZAP-70 T/B of 1.5 significantly predicted PFS in both the training and validation sets. In a Cox multivariate analysis (model 1) in which ZAP-70 I/C 40%, ZAP-70 T 40% and ZAP-70 T/B 1.5 were forced, the latter remained the sole variable maintaining an independent association with PFS in early Binet A CLL. However, when in Cox multivariate analysis all ZAP-70 procedures were forced together with IGHV mutational status (model 2), ZAP-70 T/B 1.5 lost its predictive power and IGHV mutational status remained the sole variable maintaining an independent association with PFS. Finally, three-year reassessment of ZAP-70 levels showed that among the 82 ZAP-70 I/C negative cases, 27 (32.9%) became positive, while 10 of the 30 positive cases became negative. Using the ZAP-70 T method, 23/78 (29.5%) cases that presented as negative at diagnosis, shifted to positive, while 6/34 (17.6%) positive cases became negative. Finally, with the ZAP-70 T/B FC method, 27/66 (40.1%) negative cases became positive and 16/46 (34.8%) positive cases were recorded as negative. *Conclusions.* We conclude that regardless of the detection method adopted, the independent predictive power of ZAP-70 expression for progression in early-stage CLL remained unconfirmed. Moreover, ZAP-70 expression proved relatively unstable with time.

Myelodysplastic Syndromes

PO-038

DO MYELODYSPLASTIC/MYELOPROLIFERATIVE NEOPLASMS AND MYELODYSPLASTIC SYNDROMES WITH NUCLEOPHOSMIN 1 (NPM1) MUTATIONS REALLY EXIST?

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Introduction. *De novo* AML with mutations of NPM1 gene account for about 30% of all cases, 50-60% of AML cases with normal karyotype (NK), whereas NPM1 mutations are less frequently observed in secondary AML, arising from either MDS or MDS/MPN, ranging from 8% to 17% of the cases. Furthermore, NPM1 mutations are rarely found in patients with either MDS or MDS/MPN (2%-4% of the cases), but scanty information is reported about immunohistochemical (IHC) examination for NPM1 in this setting. *Methods*. We describe 2 cases, observed with PB and BM myeloid blast count <20%. Patient 1, a 57-year old woman, was initially diagnosed with atypical CML, BCR-ABL negative, with NK. Patient 2, a 47-year old woman, was first diagnosed with MDS/MPN-unclassifiable, with NK. Surprisingly, in both patients NPM1 mutation was documented by PCR analysis. The IHC examination of BM trephine biopsy documented cytoplasmic NPM1 (NPMc+) staining in more than 20% of cells, so, in both patients, AML with mutated NPM1 was finally diagnosed and treated with intensive chemotherapy, obtaining CR and subsequent favorable outcome. We have retrospectively analyzed 175 further adults with either MDS or MDS/MPN, consecutively observed over a period of 6 years (2008-2013). Overall, BM aspirate samples were available for molecular analysis to investigate the presence of NPM1 mutations in 135 cases (76.3%). For patients with NPM1 mutations, subcellular localization of NPM1 was investigated by IHC analysis, performed on BM trephine biopsy. *Results*. A total of 177 patients, namely 140 MDS and 37 MDS/MPN (29 CMML) cases, 109 males and 68 females, of median age 77 years (range 47-93 years) were analyzed (Table 1).

Table 1. Characteristics of patients with MDS or MDS/MPN from our series.

Total number of MDS and MDS/MPN cases	177
n° of MDS cases (%)	140/177 (79)
n° of MDS/MPN cases (%)	37/177 (21)
Sex	
Male (%)	109/177 (61.5)
Female (%)	68/177 (38.5)
Median age (years)	77 (range 47-93)
MDS classification according to WHO 2008 (n° of cases)	
RCUD	6
RARS	27
RCMD	10
RAEB-1	42
RAEB-2	35
MDS-U	10
MDS associated with isolated del(5q)	4
Therapy-related MDS	6
MDS/MPN classification according to WHO 2008 (n° of cases)	
CMML	29
aCML, BCR-ABL1-negative	1
RARS-t	3
MDS/MPN-U	4
Morphological examinations (n° of cases)	
PB smear (%)	177/177 (100)
BM aspirate (%)	167/177 (94.4)
BM trephine biopsy (%)	136/177 (76.8)
Cytogenetic analyses	
Sample not available (%)	26/177 (14.7)
Normal karyotype (%)	79/151 (52.3)
Chromosomal abnormalities (%)	72/151 (47.7)
BM samples available for NPM1 mutations PCR analysis	
MDS and MDS/MPN cases (%)	135/177 (76.3)
MDS cases (%)	108/140 (77.1)
MDS/MPN cases (%)	27/37 (73)
CMML cases (%)	16/29 (55.2)
Frequency of NPM1 mutations by PCR analysis	
MDS and MDS/MPN cases (%)*	4/135 (3)
MDS/MPN cases (%)**	2/27 (7.4)
MDS cases (%)***	2/108 (1.9)
CMML cases	0/16

MDS, myelodysplastic syndromes; MDS/MPN, myelodysplastic/myeloproliferative neoplasms; RCUD, refractory cytopenia with unilineage dysplasia; RARS, refractory anemia with ring sideroblasts; RCMD, refractory cytopenia with multilineage dysplasia; RAEB-1, refractory anemia with excess of blasts-1; RAEB-2, refractory anemia with excess of blasts-2; MDS-U, myelodysplastic syndrome-unclassified; CMML, chronic myelomonocytic leukemia; aCML, atypical chronic myeloid leukemia; RARS-t, refractory anemia with ring sideroblasts and thrombocytosis; MDS/MPN-U, myelodysplastic/myeloproliferative neoplasm-unclassifiable; PB, peripheral blood; BM, bone marrow.

* In all these four cases, the immunohistochemical (IHC) examinations on BM trephine biopsies documented cytoplasmic NPM1 (NPMc+) staining in more than 20% of cells.

** In these two cases, namely patients 1 and 2, who were initially suspected as having aCML and MDS/MPN-U, respectively, NPMc+ acute myeloid leukemia was finally documented, based upon IHC examination on BM trephine biopsy.

*** IHC examination for NPM1 on BM trephine biopsy was retrospectively performed in these two deceased cases, namely patients 3 and 4, 42 and 22 months after MDS diagnosis, respectively.

BM aspirate and trephine biopsy morphologic examinations were available for 167 (94.4%) and 136 (76.8%) cases, respectively. Including patients 1 and 2, NPM1 mutations were observed in 4 out the 135 (3%) BM aspirates available for the molecular analysis. The IHC examinations, retrospectively performed on BM trephine biopsies from the 2 further elderly patients with RAEB-2 (patients 3 and 4, observed in 2010 and 2012, respectively), in whom NPM1 mutation was observed, documented NPMc+ staining in more than 20% of cells, suggesting AML with mutated NPM1. Patient 3, a 79-year old man, developed AML after 6 cycles of 5-AZA, whereas patient 4, a 85-year old woman with pancytopenia, received BSC only. *Conclusions*. Caution is needed when defining a case as NPM1-mutated MDS, since NPMc+ AML frequently shows multilineage involvement and dysplastic features. Moreover, MDS and MDS/MPN cases with NPM1 mutation frequently progress to AML, within 0.5 to 16 months since diagnosis. Our results suggest that in the rare cases of either MDS or MDS/MPN with a blast count invariably <20%, but with NPM1 mutation on molecular analysis, AML may be under-diagnosed. IHC examination may be the method of choice to precisely evaluate the percentage of BM cells with NPMc+ staining, thus reaching a definite NPMc+ AML diagnosis.

PO-039

SAFETY PROFILE OF ERWINIA ASPARAGINASE TREATMENT IN ADULTS WITH NEWLY DIAGNOSED ACUTE LYMPHOBLASTIC LEUKEMIA: A RETROSPECTIVE MONOCENTER STUDY

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Introduction. Several comparative studies in children with ALL have shown superior efficacy for E. coli Asparaginase (EcA) when compared with Erwinia Asparaginase (ErA), but at expense of increased toxicity. ErA is actually used as second or third-line treatment in patients who have developed hypersensitivity to EcA, with good tolerability and efficacy. Scanty information is reported on efficacy and toxicity in adults with newly diagnosed ALL treated according to combination regimens including ErA, as front-line treatment. *Methods*. To address this issue, we retrospectively analyzed a series of 48 adult ALL patients observed at our Institution between 2006 and 2013. Thirteen patients, of median age 32 years (range 20-58 years) received either ErA (11 cases) or EcA (2 cases) during remission induction treatment, according to three different regimens (Table 1). *Results*. All patients entered hematologic complete remission and none suffered from hypersensitivity reactions. Thrombotic complications (pulmonary embolism and superficial venous thrombosis) occurred in 2 patients (18.2%) treated with ErA, while 1 patient (50%) treated with EcA developed portal thrombosis after splenectomy. Regarding the effects on coagulation parameters, PT was prolonged (median 89%, range 49-100%), at least in one determination, in 6 subjects (54.5%) receiving ErA and in 1 (50%) treated with EcA, while aPTT was not significantly altered. Antithrombin (AT) level was <70% (median 100%, range 62-131%) in 2 (18.2%) under ErA and in all patients receiving EcA, whereas fibrinogen level was <100 mg/dl (median 217 mg/dl, range 70-887 mg/dl), only in 8 patients (72.7%) undergoing ErA. Two patients (18.2%) treated with ErA received prophylactic AT and fibrinogen concentrates, whereas in all patients receiving EcA only AT supplementation was provided. Seven patients (63.6%) treated with ErA and all those receiving EcA experienced hepatotoxicity, but only in one case ErA discontinuation was required. Pancreatic toxicity, with asymptomatic amylase and lipase elevation, was observed in only 2 cases (18.2%) after ErA, while hyperglycemia was documented in 3 (27.3%) patients under ErA and 1 (50%) after EcA, during concomitant corticosteroid treatment. At a median follow-up of 31 months (range 6-65 months), 10 patients are alive. Two deaths occurred for leukemia relapse and one because of invasive aspergillosis. *Conclusions*. In our small and heterogeneous cohort of adults with ALL treated in first-line with regimens including either ErA or EcA, no unexpected toxicities were identified and the treatments were generally well tolerated. Even if we cannot make any direct comparison in terms of efficacy and toxicity profiles from our series, we could suggest that remission induction chemotherapy regimens with

ErA in first-line may be feasible in adult patients with previously untreated ALL. Future perspective randomized clinical trials may be warranted to compare efficacy and safety of the two asparaginase preparations.

Table 1. Clinical and laboratory information on adult patients with acute lymphoblastic leukemia (ALL) treated with L-asparaginase.

Pt	Age (ys)/ Sex	Diagnosis/ Treatment schedule	L-asp	Thrombosis/ hemorrhage	CVC	PT (%) Diagnosis/ before 1st L-asp dose/ median/ range	Fibrinogen (mg/dl) Diagnosis/ before 1st L-asp dose/ median/ range	AT (%) Diagnosis/ before 1st L-asp dose/ median/ range	Plt ($x10^9$/L) Diagnosis/ before 1st L-asp dose/ median/ range	AT / fibrinogen Concentrates (n° of infusions)	Albumin and total proteins	Hepatic toxicity (WHO grade)	Pancreatic toxicity (WHO grade)	Glycemia	Hypersensitivity
1	25/M	T-ALL/A	Erwinia	No/No	PICC	74/100/ 88/55-100	361/112/ 203/72-803	110/110/ 107/100-119	481/152/ 206/109-537	No/No	Normal	Yes (grade 3)	No	Normal	No
2	21/M	T-ALL/B	Erwinia	No/No	PICC	77/81/ 81/66-100	234/180/ 190/111-283	113/113/ 98/83-114	12/19/ 142/12-422	No/No	Normal	No	No	Normal	No
3	20/M	B-ALL/B	Erwinia	Pulmonary embolism/No	No	84/89/ 77/49-100	321/182/ 263/70-612	113/126/ 100/63-128	36/19/ 76/7-376	Yes (1) / Yes (1)	Normal	Yes (grade 3)	Yes (grade 2)	Normal	No
4	51/M	B-ALL/A	Erwinia	SVT/No	No	100/97/ 94/79-100	183/104/ 136/81-521	109/109/ 97/88-109	159/44/ 71/13-159	No/No	Normal	Yes (grade 3)	Yes (grade 2)	Increased	No
5	49/M	T-ALL/A	Erwinia	No/No	PICC	87/80/ 89/65-100	411/95/ 191/79-593	98/98/ 100/83-128	86/18/ 68/9-118	No/No	Normal	Yes (grade 3)	No	Normal	No
6	27/M	T-ALL/B	Erwinia	No/No	PICC	59/87/ 89/51-100	165/122/ 158/85-277	123/113/ 106/91-125	101/204/ 134/66-264	No/No	Normal	No	No	Normal	No
7	57/M	B-ALL/A	Erwinia	No/No	PICC	100/100/ 100/92-100	442/193/ 256/84-692	87/94/ 98/77-113	8/25/ 57/8-183	No/No	Normal	No	No	Increased	No
8	21/M	B-ALL/B	Erwinia	No/No	PICC	100/93/ 98/86-100	318/170/ 171/87-408	116/116/ 117/103-131	251/234/ 158/57-315	No/No	Normal	Yes (grade 3)	No	Normal	No
9	32/M	B-ALL/A	Erwinia	No/No	No	78/100/ 99/78-100	589/177/ 217/112-589	100/100/ 110/100-120	491/145/ 234/57-491	No/No	Normal	Yes (grade 3)	No	Normal	No
10	23/F	T-ALL/B	Erwinia	No/No	PICC	66/66/ 64/50-75	487/132/ 302/78-887	93/120/ 96/63-132	126/93/ 113/11-555	Yes (4) / Yes (4)	Decreased (2 g/dl/ 3.8 g/dl)	Yes (grade 4)	No	Increased	No
11	38/F	T-ALL/A	Erwinia	No/No	No	85/100/ 98/78-100	510/158/ 301/132-620	108/124/ 114/105-120	113/388/ 175/57-479	No/No	Normal	No	No	Normal	No
12	58/M	B-ALL/C	E. coli	No/No	No	100/68/ 85/68-100	409/155/ 250/140-458	85/110/ 77/62-110	99/17/ 57/12-133	Yes (3) / No	Normal	Yes (grade 2)	No	Increased	No
13	42/F	T-ALL/C	E. coli	Portal thrombosis after splenectomy/No	PICC	74/100/ 93/74-100	536/269/ 356/111-693	90/64/ 84/64-107	138/171/ 277/12-646	Yes (2) / No	Normal	Yes (grade 3)	No	Normal	No

Pt, patient; L-asp, L-asparaginase; SVT, superficial venous thrombosis; CVC, central venous catheter; PICC, peripherally inserted central catheter; PT, prothrombin time; AT, antithrombin; Plt, platelet count, WHO, World Health Organization.

Remission induction chemotherapy regimens.
Treatment schedule A: prednisone pretreatment and then prednisone 60 mg/m^2 from day +1 to day +24 and then tapering; vincristine (VCR) 1.4 mg/m^2 on days 1,8,15,22,36; daunorubicin (DNM) 30 mg/m^2 on days 1-3, 22-24, 36-38; Erwinia L-asparaginase 6000 IU/m^2 intravenously 3 times a week starting from day +10 for a total of 10 doses.
Treatment schedule B (cycle Ia): pretreatment with prednisone 60 mg/m^2 from day +1 to day +7; prednisone 60 mg/m^2 from day +8 to day +28 then tapering for B-ALL OR dexamethasone 10 mg/m^2 on days 8-14 and 22-28, then tapering for T-ALL; VCR 1.5 mg/m^2 on days 8,15,22,29; DNM 30 mg/m^2 on days 8,15,22,29; Erwinia L-asparaginase 10000 IU/m^2 intravenously on days 12,15,18,21,24,27,30,33 for a total of 8 doses.
Treatment schedule C: pretreatment with prednisone 20 mg/m^2 every 12 hours on days -5 to -1 and cyclophosphamide 300 mg/m^2 on from day -3 to day -1; VCR 1.4 mg/m^2 on days 1,8,15,22; idarubicin 12 mg/m^2 on days 1,2; dexamethasone 5 mg/m^2 every 12 hours on days 1-5 and 15-19; L-asparaginase from E. coli 3000 IU/m^2 intravenously on days 8,10,12,15,17,19 for a total of 6 doses; filgrastim 5 mcg/kg/day from day +5 to neutrophil count recovery.

PO-040

ATP INDUCES APOPTOSIS IN AML CELLS AND POTENTIATES THE CYTOTOXIC EFFECT OF CYTARABINE ACTING IN A SYNERGISITIC WAY

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Introduction. It is becoming increasingly evident that tumor onset and progression depend not only on properties intrinsic to cancer cells. Both the biochemical composition of the tumor microenvironment and the cancer-host interaction appear to be important in disease evolution. Extracellular nucleotides may now acquire a new biologic significance as part of the complex molecular miscellany composing the tumor environment. Over the past decade, several *in vitro* studies described ATP capability of suppressing cell growth in several tumor cells. In line with these findings, our group recently investigated the role of purinergic signaling in the tumor microenvironment associated with acute myeloid leukemia (AML). *Methods.* ATP and ARA-C antineoplastic effects were analyzed by inhibition of proliferation (MTS assay) and induction of apoptosis (Annexin V/PI staining, Caspase 3 activity). Leukemia cell lines were co-cultured with human mesenchymal stromal cells and antineoplastic effects of ATP or ARA-C were then measured. For *in vivo* engraftment studies, NSG mice were used. *Results.* We demonstrated that extracellular ATP induces apoptosis of leukemia cell lines via caspase-3 activation and the pro-apoptotic effect is directly correlated with P2X7R expression on leukemia cells. In addition, we investigated whether the combination of Ara-C cytotoxicity with the ATP-induced apoptosis interacts in a synergistic way to enhance their antineoplastic activity. Our data show that ATP in combination with cytarabine potentiates the cytotoxic effect of chemotherapy, significantly reducing cell proliferation and increasing apoptosis of AML cell lines. A growing amount of evidence suggests that bone marrow microenvironment, in particular mesenchymal stromal cells (MSCs), supports leukemia cell survival and may provide protection against chemotherapy, included Ara-C. We evaluated whether MSCs are able to attenuate the antineoplastic effect of ATP on leukemic cells. MSCs don't mediate protection of AML cells from ATP induced apoptosis and the synergistic effect of ATP and ARA-C is preserved also in presence of stroma layer. Moreover, preliminary *in vivo* experiments showed that ATP antineoplastic effect observed *in vitro* also occurs *in vivo*. Intraperitoneal injections of ATP significantly reduce human leukemia cell engraftment in immunodeficient mice. *Conclusions.* Taken together, our data suggest that ATP has an anti-leukemic activity and this action seems to be mediated through P2X7 receptor. The results show that it could be used as adjuvant to potentiate the cytotoxic effect of chemotherapic drugs reducing their concentration. The finding that ATP effectively reduces the growth of AML cells *in vitro* and *in vivo* highlights the potential use of ATP in the treatment of leukemia.

PO-041

ALTERNATIVE SPLICING OF HTERT EXON 7: A FURTHER MECHANISM FOR THE CONTROL OF ACTIVE HTERT ISOFORMS EXPRESSION

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Telomerase is a ribonucleoprotein complex with a reverse transcriptase (hTERT), a key regulator of this enzyme. The existence of the alternatively spliced variants of hTERT suggests complex regulation of this gene in normal development. The main known hTERT isoforms are the full-length hTERT transcript (+α+β) and those carrying α and/or β deletions (transdominant negative isoform -α+β, inactive products +α-β and -α-β) (Kilian *et al.* 1997). Aims of our study were to estimate the distribuition of the hTERT isoforms in a series of *de novo* AML patients and to correlate expression levels with chromosomal complexity, *i.e.* with normal *versus* complex karyotypes. The ninety-seven *de novo* AML patients were diagnosed at our Institution between January 2010 and January 2013. They were thirty-eight females and fifty-nine males; their median age was 59 years (range 18-84). On conventional cytogenetic studies fifty-two presented a normal karyotype and forty-five presented a complex karyotype. hTERT isoforms expression was determined in bone marrow samples by real-time reverse transcriptase polymerase chain reaction, using SYBR Green I. hTERT transcript (+α+β) primers' design was made using BLAST, while for the other primers we referred to Capraro *et al.* 2011. Specific amplifications were confirmed by sequences analysis. In order, to estimate hTERT isoforms expression levels in normal mononuclear cells, twenty-three umbilical cord bloods (UCB) were examined. When the Kruskal-Wallis rank test was applied to compare hTERT isoforms expression levels between chromosomally normal AML and chromosomally complex AML no significant difference was observed (P=0.39, P=0.77; +α+β, -α+β respectively). Instead, independently from karyotype, in Cox univariate analysis, we observed that patients with a high trans-dominant negative isoform expression had a significant better response to treatment than the other patients (P=0.03, HR=2.11; 95% CI:1,13-3,93). In addition, a significant inferior leukemia-free survival was observed in patients who presented a high expression of inactive products (-α-β) when compared to those who presented a low expression (P=0,003, HR=3,26; 95% CI:1,33-7,96). Patients who presented a high expression of the full-length hTERT transcript and the trans-dominant negative isoform presented only a trend towards significance. The first group of patients presented a worse overall survival (HR=1,66; 95% CI:0.94-2,91), whereas the second group a better overall survival (HR=0,58; 95% CI:0,33-1,02). We conclude that a high expression of the trans-dominant negative isoform can identify low-risk AML patients, while a high expression of the inactive product (-α-β) can identify high-risk disease patients. In addition, we suggest that this last isoform, an alternatively spliced variant of hTERT, may control the expression of the trans-dominant negative isoform. Our results suggest an intriguing link between the control of hTERT isoforms expression and AML outcome.

PO-042

THE INTRODUCTION OF BIOLOGICAL FEATURES AT BASELINE IMPROVES THE RELAPSE RISK STRATIFICATION IN ACUTE PROMYELOCYTIC LEUKEMIA PATIENTS

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Acute promyelocytic leukemia (APL) patients can be stratified into three different subgroups - low, intermediate and high risk – on the basis of the WBC and platelet counts according to the PETHEMA criteria. A risk-adapted strategy based on this classification has improved the clinical outcome of APL patients, as previously reported. We hereby investigated in a multivariate analysis the clinico-biological features of patients at baseline in an attempt to identify other prognostic features

and improve the previously reported PETHEMA score. Our database included 143 newly diagnosed APL patients. In all cases, the diagnosis was confirmed by RT-PCR identification of the disease specific PML/RARA fusion gene. Seventy-three patients (January 1993-May 2000) received uniform post-remission treatment based on the AIDA 0493 regimen, while 69 patients (diagnosed after May 2000) were treated according to the risk-adapted regimen AIDA-2000. Using the PETHEMA relapse risk scoring system, 34 patients were classified as low risk, 67 as intermediate and 42 as high risk. The following characteristics were tested in multivariate analysis: age, sex, relapse risk (which included WBC and platelet counts), hemoglobin level, type of transcript, FlT3-ITD mutation, morphologic subtype, presence of aberrant phenotypic antigens (CD34, CD15, CD56), coagulopathy. We identified that the type of transcript, FlT3-ITD mutation, morphologic subtype and presence of CD34 maintained an independent prognostic value. We accordingly constructed a predictive model that stratified patients into three categories: 1) low risk: presenting with 0 or only 1 risk factor; 2) intermediate risk: 2 o 3 risk factors; 3) high risk: presenting with 4 to 6 risk factors. The numerical distribution of patients into the above three groups was as follows: low risk 34 patients (24%), intermediate risk 67 patients (47%) and high risk 42 patients (29%). With the introduction of biological parameters, we identified a statistical difference in terms of overall survival (OS) (96% for low risk, 87% for intermediate and 60% for high risk patients, p=0.02), relapse and disease-free survival (92.5% for low risk, 76% for intermediate and 58.4% for high risk category, p=0.001). The new stratification is also associated with a significant difference in the prevalence of differentiation syndrome: 9.4% for low risk, 14.8% for intermediate and 22% for high risk, p=0.002). Most importantly, this new prognostic model seems capable of identifying patients with a low OS likelihood. Further investigations are required in order to validate this model on an independent series of APL patients.

PO-043

LIN28 EXPRESSION IN ACUTE MYELOID LEUKEMIA

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Introduction. AML is a heterogeneous disorder of hematopoietic progenitors. Although some pathways involved in the maintenance of leukemic stem cells have been identified (Wnt, Notch), a successful targeted therapy is not available yet. To improve our current knowledge on the biology of leukemic processes represents a way to identify new potential drug targets or molecular diagnostic/prognostic markers. Lin28 is a conserved RNA-binding protein playing an important role in cancer stem cells. It has been reported the existence of reciprocal regulatory loops between Lin28 and several miRNAs. In murine model overexpression of miR-125b leads to a down-regulation of Lin28 and a preleukemic state characterized by overproduction of myeloid cells eventually progressing to a myeloid leukemia. Conversely, ectopic expression of Lin28 reprograms hematopoietic progenitor cells from adult bone marrow (BM), endowing them to mediate multi-lineage reconstitution which resembles fetal lymphopoiesis. Since Lin28 seems to be an important regulator of hematopoiesis, it could be interesting to study its involvement in the induction and maintenance of AML. This could help to better characterize the behavior and the mechanisms of chemoresistance of leukemic cells (LC). Aim of this study is to investigate the role of Lin28 and its potential regulatory circuits in AML LC. *Methods.* BM blood specimens were collected from 32 *de novo* AML, 3 secondary AML and 9 healthy donors used as controls. CD34+ cells were separated from healthy control. We analyzed, by qRT-PCR, Lin28A and Lin28B expression levels in AML patients, healthy samples and AML cell lines (Kasumi1 and OCI-AML3). Lin28 protein expression was evaluated by cytofluorimetric analysis in myeloid (CD34+CD45+CD33+, CD34+CD45+CD71+) and lymphoid (CD34+CD45+CD19+) precursors of 6 BM healthy subjects and in 6 AML LC. *Results.* Real Time data indicate a down-regulation of Lin28A (p=0,0003) in AML patients as compared with normal CD34+ progenitor cells; by contrast, Lin28B was not expressed in AML. The differences of Lin28A expression, among stratified patients for genetic mutations (WT1, NPM1, FLT3), were not statistically significant. Since Lin28 seems to be involved in hematopoiesis, we studied Lin28A protein expression in myeloid and lymphoid precursors of normal BM subjects. Our data showed an up-regulation of Lin28A in myeloid precursors with respect to the lymphoid ones ($p<0.05$). To confirm the qRT-PCR data, we analyzed Lin28A protein expression in AML LC as compared with normal myeloid precursors, showing a down-regulation of Lin28A protein ($p<0.05$) like mRNA levels. *Conclusions.* We show here for the first time a down-regulation of Lin28A in cancer patients; this is in contrast with current literature data that show its up-regulation in other neoplastic disorder. Furthermore, our data indicate Lin28 as a hypothetical regulator of myeloid differentiation suggesting its possible involvement in AML leukemia.

PO-044

TOSEDOSTAT PLUS LOW DOSE CYTARABINE INDUCES A HIGH RATE OF RESPONSES IN PREVIOUSLY UNTREATED OLDER PATIENTS WITH ACUTE MYELOID LEUKEMIA (AML): A PHASE II MULTICENTER STUDY

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Introduction. Outcome for older patients with acute myeloid leukemia (AML) is extremely poor. Furthermore, intensive induction chemotherapy is often unsuitable. Tosedostat is a new, orally bioavailable inhibitor of members of the M1 and M17 classes of aminopeptidases that includes the zinc-dependent aminopeptidases. Tosedostat was proven to be effective as single agent, with a manageable safety profile, in both *de novo* and relapsed AML. We hypothesize that adding todesostat to cytarabine may improve the response rate and remission duration over what is expected with chemotherapy or tosedostat alone. *Methods.* This is a phase II, prospective, multicenter study, designed according to Fleming's method. Fixing the lowest acceptable rate as 10% and the successful rate as 25%, with a significance level aalpha=0.05 and a power 1-beta=0.80, the sample size was estimated in 33 patients. Twenty-nine patients were enrolled so far. Here we report the results on 22 evaluable patients (7 patients are too early). All patients (median age 75 years) received Tosedostat 120 milligrams orally once daily from day 1 to 240, coupled with intermittent cytarabine given subcutaneously at 20 milligrams twice/day from day 1 to day 10 of each cycle. Courses of cytarabine were repeated every 4 weeks in the absence of disease progression or unacceptable toxicity, up to 8 cycles. *Results.* Median white blood cell count at diagnosis was 2.6x10^9/l (range: 0,7-21.4x10^9/l). 17/29 patients had an intermediate karyotype, 8/29 an unfavorable karyotype and 4/29 were not evaluable. Thirteen patients had a *de novo* AML, whereas 16 patients had a secondary AML. Induction-period mortality was 7%, with 2 deaths occurring in aplasia during cycle 1. Overall CR rate was 40% among evaluable patients (8/20). Interestingly, 4 additional patients obtained or a partial response (2) or a stable disease, translating in an impressing overall response rate of 60%. Eight patients did not respond at all to therapy and rapidly died with progressive disease after having received 2 cycles of cytarabine and 45 days of tosedostat. In responding patients, the median time for best response was 61 days (range 22-145). Four out of 12 responding patients are still in CR after a median follow-up of 7 months (range: 3-13), whereas 2/12 are in PR after 4 and 5 months, respectively and 2/12 are alive, with stable disease. One patient died in CR due to an infectious complications, whereas 11 patients died due to leukemia (8 refractory, 3 relapsed). *Conclusions.* The combination of tosedostat and low-dose cytarabine induce a high rate of responses in a subset of elderly AML patients with

extremely poor-prognosis, not suitable for intensive chemotherapy. The study was registered at EMA with the EUDRACT number 2012-000334-19. *Acknowledgements.* Chroma is gratefully acknowledged for providing Tosedosatat for the patients. The study was supported in part by AIL Pesaro Onlus.

PO-045

EXPRESSION AND PROGNOSTIC ROLE OF MENINGIOMA-1 (MN1) GENE EXPRESSION AND CORRELATION WITH NPM1 MUTATION AND BAALC EXPRESSION IN PATIENT WITH ACUTE MYELOID LEUKEMIA

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Introduction. Molecular markers are necessary for prognostic stratification and monitoring of minimal residual disease in acute myeloid leukemia (AML). Cytogenetic aberrations have long been recognized as the most important prognostic variable in AML, and are still the major determinant for post-remission therapy. Unfortunately, only 50-60% of AML patients presents an abnormal karyotype. Meningioma 1 (MN1) gene has been found to be over-expressed in AML with inv(16), and high MN1 levels seems to have prognostic impact in cytogenetically normal AML patients. Others molecular markers are known to have a prognostic role in AML, such as nucleophosmin (NPM1) gene mutation and expression of brain and acute leukemia, cytoplasmic (BAALC) gene. To test the possible prognostic role of MN1 in AML, irrespectively of karyotype status, we studied MN1 expression in a cohort of 171 consecutive AML patients, treated at the Division of Hematology of Udine between 2004 and 2013. We also compared the expression of MN1 with mutation of NPM1 and expression of BAALC. *Methods*. To assess MN1 expression, we designed a quantitative PCR assay with TaqMan chemistry, using ABL as housekeeping gene. To determine the relative quantification of MN1 expression, we used the 2-ΔΔCt method, based on a calibrator obtained on a control group of 12 healthy donors. Results MN1 resulted expressed in all the tested cases, with a median value of 1.1, that was thus considered the cut-off for defining positive (MN1+) and negative (MN1-) cases. The MN1+ cases was not significantly different across the various cytogenetic subgroups (7/10, 70% in favourable karyotype, 38/84, 45% in intermediate karyotype and 22/37, 59% in unfavourable karyotype), but expression level of MN1+ cases was significantly higher in unfavourable (median 7.0, range 1.2-64.7), compared to intermediate (median 2.98, range 1.17-61.9, p=0.007) and favourable (median 1.9, range 1.1-49.9, p=0.01). High MN1 expression was associated with lower frequency of NPM1 mutations (5/70 *vs* 31/71, p=0.0001) and higher BAALC expression (30/68 *vs* 7/69, p=0.0001); no other clinical or biological characteristics were significantly associated with MN1 expression. Complete Remission (CR) rate of the entire cohort was 58%, and MN1 status was not associated with CR achievement. With a median follow-up time of 12 (range: 1-95 months), 3-years disease free survival (DFS) and overall survival (OS) were 45% and 32%, respectively. MN1 expression per se had no impact on DFS and OS; however, considering MN1 in association with other molecular markers, we found that MN1- cases with NPM1 mutation and low BAALC expression had a significantly longer 3-years OS (51% *vs* 28%, p=0.05) (Figure 1).

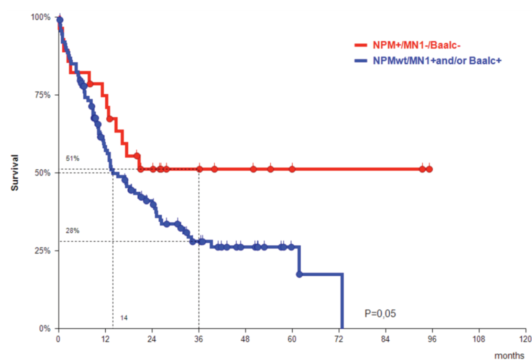

Figure 1. Low expression of MN1 (MN1-), BAALC (Baalc-) and mutated NPM1 (NPM+) predict longer Overall Survival.

Conclusions. The MN1 status, in our cohort, is not associated with a specific genetic subset and do not represent a prognostic factor in terms of CR and OS. On the other hand, MN1 seems identify a defined subgroup with better prognosis in association with NPM1 and BAALC.

PO-046

MYELODYSPLASTIC SYNDROMES: HOW AMELIORATE THE ACCURACY OF DIAGNOSIS BY APPLYING AN INTEGRATED MOLECULAR/CYTOGENETIC WORKUP

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In MDS, cytogenetics is fundamental for the risk stratification, but in this setting non-informative karyotypes represent up to 15-20% of cases. The aCGH is able to detect new abnormalities in up to 80% of cases already tested by conventional karyotype, but it is not enclosed in the routinary diagnostic workup. More recently, the whole genome sequencing methods detected in MDS relevant mutations involving TET2, ASXL1, EZH2, CBL, IDH1/IDH2, DNMT3A. TET2 mutations have been related to a better survival in patients receiving 5-azacitidine, whereas ASXL1, TP53, and EZH2 mutations have been associated with worse outcome. High WT1 expression levels have been related to shorter OS. In this study, we assessed 50 new MDS cases by different techniques: a) conventional cytogenetics; b) FISH for chromosome 5, 7, PDGFRA, and PDGFRB rearrangements; c) aCGH; d)real-time PCR assay for ASXL1, EZH2, TP53, and TET2 mutations. The aim of the study was to determine the adjuctive value offered by FISH, aCGH, and somatic mutation assays in respect of the conventional cytogenetics and to determine if the proposed new diagnostic workup will reach good sensitivity and specificity, necessary for a routinary application. After Giemsa banding, one third of our samples showed chromosomal aberrations, including +8, del(7), del(5), -Y, +6, del(13), +14, del(20), and complex karyotypes. After the FISH analysis, 17% of patients showed chromosomal abnormalities, including 5q- and del(13) that were not detected by the Giemsa banding. The aGCH allowed to detect quantitative chromosomal aberrations in 46% of cases (del(13), -7, del(12), del(16), del(17), del(11), del(8), dupl(14), 5q-). After the RT-PCR assessment, 22% of patients resulted mutated for TP53 gene; the involved nucleotides were 844 (C>T), 733 (G>A), 742 (C>T), and 853 (G>A). Four of these TP53 mutated patients showed normal karyotype, and resulted unmutated also by FISH and aCGH . The WT1 gene was over-expressed (in comparison to healthy subjects) in the 25% of the assessed cases; 50% of these patients presented with RAEB and IPSS intermediate-2/high and had a worse outcome. The half of these patients had a normal karyotype. The RPS14 gene was under-expressed in 77% of cases, analogously to the percentage already reported by our group. Clinical correlations and evaluation of the outcome of these patients are being performed at this time, but we already reported that lower RPS14 and higher WT1 levels did negatively impact on the outcome. In conclusion, these data sustain the fundamental role of the integrated diagnostic work-up for MDS: indeed, 2 cases, correctly classified as affected by the 5q- syndrome after the FISH analysis, received lenalidomide. In one third of the remaining cases, the identification of TP53 and ASXL1 mutations, in addition to the abnormalities of chromosome 17, 8, 11, and 16 detected after aCGH, allowed us to scoring patients as at higher risk. These patients are now candidate to receive azacitidine.

PO-047

AKT PROMOTES THE ONCOGENIC NOTCH SIGNALING IN T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA

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Introduction. Notch and AKT/PI3K signaling are two key oncogenic pathways closely associated in T-cell acute lymphoblastic leukemia (T-ALL). These pathways collaborate in controlling proliferation, survival

and migration of T-ALL cells and are deregulated in 60% (Notch pathway) and 48%(AKT/PI3K) of T-ALL patients. Recent evidences indicate that, in T-ALL cells, Notch and AKT/PI3K pathways collaborate through a reciprocal positive control. Here we identified a novel way by which AKT regulates Notch1 activity and investigated the underlying mechanism. *Methods.* T-ALL cell line, Molt4, and HEK293T cell line were grown in RPMI-1640 and DMEM respectively, supplemented with 10% heat-inactivated FBS. 1 ug RNA isolated from cells was retro-transcribed in 20 ul by M-MuLV reverse transcriptase using random hexamer primers. RT-PCR analysis was performed using primers for Notch1, HES1, preTCRa, GAPDH. Apoptotic cells were identified by Annexin-V and propidium iodide staining. Protein expression was detected by Western blot analysis of whole cell lysates. Immunoprecipitation (IP) of ubiquitin-conjugated proteins was performed using the UbiQapture-Q Kit (Biomol, Exeter, UK), as described by the manufacturer. Co-immunoprecipitation (Co-IP) analysis was performed using Protein G Agarose beads, eluted immunoprecipitates were analyzed by Western blot. Immunofluorescent staining was done on HEK293T cells incubated with anti-Flag or anti-c-Cbl primary antibodies and the appropriate AlexaFluor-conjugated secondary antibodies. Images were acquired with a Leica TCS SP2 confocal microscope. A colocalization area was determined based on a 2D cytofluorogram and density analysis performed by Multicolor Analysis Leica Confocal software. *Results.* The influence of AKT signaling on Notch1 levels was investigated by an inhibitory approach using the PI3K inhibitor LY294002. The LY294002-mediated withdrawal of AKT reduced Notch1 protein levels and activity, without affecting Notch1 transcript. We showed that Notch1 protein decrease was due to lysosomal degradation of the Notch1 membrane-bound form. IP and Co-IP analyses revealed that AKT withdrawal resulted in an increased tyrosine phosphorylation of Notch1 followed by binding to an E3 ubiquitin ligase, c-Cbl, that mono-ubiquitinated Notch1 directing it to lysosomal degradation. *Conclusions.* To our knowledge, these provide the first evidence of mechanism by which AKT pathway controls Notch1 activity reducing the amount of protein undergoing to lysosomal degradation. Given the crucial role of Notch1 in T-ALL, our findings suggest that hyperactive AKT signaling in T-ALL may contribute to increase the oncogenic Notch signaling in T-ALL independently from mutations in Notch1.

PO-048

ROLE OF FLOW-CYTOMETRIC IMMUNOPHENOTYPING IN PREDICTION OF PHILADELPHIA CHROMOSOME IN ACUTE LYMPHOBLASTIC LEUKEMIA IN ADULTS

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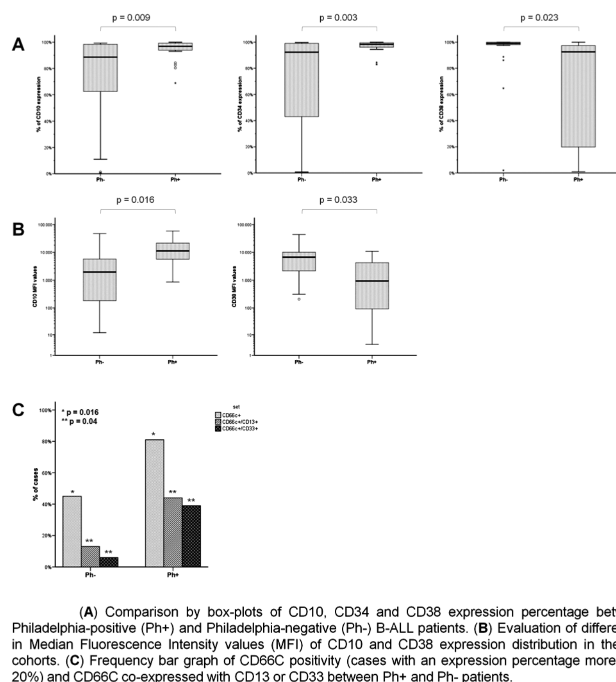
Introduction. Among B-cell precursor acute lymphoblastic leukemia (B-ALL), t(9;22) (q34;q11) responsible for BCR/ABL1 fusion transcripts is the most common cytogenetic abnormality that occurs in around 20-30% of adult patients (pts). Philadelphia-positive (Ph+) B-ALL has been typically associated with high expression of myeloid antigen such as CD13, CD33, CD66c, CD25. We analyzed immunophenotypic pathway of 44 adult patients with B-ALL diagnosed in our center from December 2004 to December 2013, with a median age of 46 ys (range 13-78), 23 males and 21 females. *Methods.* Immunophenotyping was performed using a standard lyse/wash technique. Antibodies definition panel: FITC-CD45; PE-Cy7-CD19; APC-HLA-DR; FITC-CD20; PE-CD22; FITC-CD58; PE-CD10; APC-CD34; PE-CD13; APC-CD33; FITC-CD66c; APC-CD38; FITC-CD24; FITC-NUTdT; PE-NG2; FITC-CD65 FITC-CD15 FITC-MPO PE-cyCD79A. Data were acquired on FACSCanto cytometer using BDFACSCanto software (BD Biosciences). Levels of BCR-ABL fusion transcript were quantified in a multiplex RT-PCR assay. Data were analyzed using SPSS 15.0 version. As the low sample size, the statistical analysis were performed by using both parametric (Student's t-test) and non-parametric (Mann-Whitney) tests. Reported p-values showed a comparable significance in both type of statistics. The Chi-squared test or Fisher exact test were applied for categorical variables. Arbitrary cutoff of 20% analyzed events that were brighter than the control stain, was required for an antigen to be considered positive. *Results*. BCR/ABL transcript was identified in 21 pts (47.7%). Patients with Ph+ ALL were significantly older than their counterpart (mean age, 52 *vs* 39 ys, p=0.015). The mean leukocyte count at diagnosis was higher in the Ph+ pts than others (48.771 *vs* 11.325 x 10^9/L, p=0.010). Ph+ pts had a greater mean percentage of CD10 (94% *vs* 72%, p=0.009), and CD34 (96% *vs* 69%, p=0.003) expression, and a lower mean percentage of CD38 expression (68% *vs* 92%, p=0.023). Considering Median Fluorescence Intensity (MFI), we confirmed that Ph+ pts presented an higher CD10 MFI (mean MFI, 18059 *vs* 6285, p=0.016) and a lower CD38 MFI (3201 *vs* 8012, p=0.033) than their counterpart. CD66c positivity was more frequent in Ph+ pts than in the others (81% *vs* 45%, p=0.016). The co-expression of CD66c with CD13 or CD33 was more frequently present in Ph+ pts (44% and 39%, respectively) than in other cases (13% and 6%, respectively)(p=0,04). The sensitivity of CD66c as sole marker was 81%. The co-expression of CD66c and CD13 or CD33 presented a greater specificity (88% and 94%, respectively) and a positive predictive value (80% and 88%, respectively), Figure 1. *Conclusions*. Based on our analysis, we suggest a possible screening panel for Ph-positive prediction on B-ALL pts by using the combination of CD38, CD10, CD34, CD66C, CD13 and CD33 expressions. These data need to be confirmed by a larger sample size, in order to establish also a possible prognostic role of these markers.



(**A**) Comparison by box-plots of CD10, CD34 and CD38 expression percentage between Philadelphia-positive (Ph+) and Philadelphia-negative (Ph-) B-ALL patients. (**B**) Evaluation of differences in Median Fluorescence Intensity values (MFI) of CD10 and CD38 expression distribution in the two cohorts. (**C**) Frequency bar graph of CD66C positivity (cases with an expression percentage more than 20%) and CD66C co-expressed with CD13 or CD33 between Ph+ and Ph- patients.

Figure 1.

PO-049

AZACITIDINE IN HIGH-RISK MYELODYSPLASTIC SYNDROMES: RETROSPECTIVE ANALYSIS OF 28 PATIENTS TREATED WITH THE AZA 5-2-5 REGIMEN

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Introduction. The currently approved azacitidine (AZA) regimen for myelodysplastic syndromes (MDS) is 75 mg/sqm/die subcutaneously (SC) or intravenously (IV) for 7 days every 28 days. Tree different AZA dosing regimens, which avoid week-end dosing, have shown to induce therapeutic responses consistent with the currently approved schedule (Lyons, 2009). However, the community-based study of Lyons mainly involved lower-risk MDS patients (pts). These data prompted us to investigate the therapeutic effect of the more convenient AZA 5-2-5 regimen (50 mg/m2/d subcutaneously for 5 days, followed by 2 days no treatment, then 50 mg/m2/d for 5 days) in higher-risk MDS pts (*i.e.*: IPSS risk: high or intermediate-2). *Methods.* From December 2007, in our Institution, 28 IPSS high-or-intermediate-2 risk MDS pts. (20 males), with a median age of 70 (37-83) yrs, were treated with the AZA 5-2-5 regimen. Moreover, as our group (Follo, 2009) previously demonstrated that phosphoinositide-phospholipase C (PI-PLC) beta1, may repre-

sent a target for AZA, we quantified the degree of PI-PLCbeta1 methylation and gene expression before and during AZA administration in this group of pts and in a control group of high-risk pts previously treated with the conventional AZA regimen. *Results.* At AZA onset, IPSS risk was: intermediate-2: 22 pts; high: 3 pts; 3 pts, with intermediate-1 IPSS risk, showed other high-risk features (therapy-related MDS: 2 pts; complex karyotype: 1 pt). IPSS cytogenetic risk was: low: 17 pts; intermediate: 1 pts; high: 10 pts (8 with complex karyotypes and 2 with isolated -7 or 7q-). Revised IPSS (Greenberg, 2012) was: intermediate: 1 pt; high: 6 pts; very high: 21 pts. The pts received a median number of 8 (1- 32) AZA cycles. 26 pts (89.6%) received at least 6 cycles of AZA and were considered evaluable for response, while 2 pts prematurely discontinued AZA because of toxicity or worsening of their clinical conditions. Among the 26 evaluable pts, 20 (76.9 %) showed a favourable response, following IWG criteria (Cheson, 2006): 5 (19.2 %) Complete Remission (CR), and 15 (57.7%) Hematologic Improvement (HI). First response occurred after a median of 3 (2-7) cycles. 3 pts maintained a stable disease (SD), while the 3 remaining pts showed treatment failure. A significant toxicity (grade > 2) was observed in 10 (35.7 %) pts. The median duration of response was 7 (2-54) months. Six pts showed a long-lasting response (≥ 20 months). The median OS from the start of AZA was 16.5 (5-58) months. 24 pts died, 13 because of evolution into Acute Myeloid Leukemia, and 11 for other causes (infection, haemorrhage, heart failure, stroke, suicide). PI-PLCbeta1 methylation and gene expression appeared to be related to the therapeutic response, but not to the dose schedule. *Conclusions.* Our data seem to confirm, in a population of high-risk MDS, the effectiveness, in terms of hematologic and molecular response, of the more convenient AZA 5-2-5 regimen.

PO-050

LONG-TERM OUTCOME OF HIGHER-RISK MDS PATIENTS TREATED WITH AZACITIDINE: SINGLE CENTRE EXPERIENCE

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Introduction. The primary goals of treatment for patients with higher-risk MDS are to improve bone marrow function, suppress AML transformation and prolong survival. The international phase 3 trial AZA-001 established that azacitidine (AZA) significantly prolong median overall survival and significantly delay median time to AML transformation as compared with conventional care regimens. AZA was also associated with significant improvements in other clinically relevant outcomes, including reducing the need for transfusion, hospitalization and intravenous antimicrobials, and improving quality of life. Although several clinical trials have examined the safety of AZA, limited data are available about the long-term use of this drug. We report a retrospective analysis of the outcome of 21 higher-risk MDS patients (M/F: 10/11) treated at our Institution with AZA, from January 2008 to January 2014, with a median follow-up of 35 months (range 24-60) after the start of AZA to determine the characteristics of this subset of long-term survivors. *Methods.* Median age at diagnosis was 68 years (range 51-80) and PS was <2 in 13 cases (62%). Diagnosis according to the 2008 WHO Classification was refractory cytopenia with multilineage dysplasia in 2 cases, refractory anemia with excess blasts (RAEB)-1 in 7 cases, RAEB-2 in 12 cases. Cytogenetic risk was low in 10 cases (47.6 %; all with normal karyotype), intermediate in 2 (9.5%) and high in 9 cases (42.8%; all with complex abnormalities). All patients received AZA administered subcutaneously at a dosage of 75 mg/m2 daily for 7 days, repeating the cycle every 28 days; median number of AZA cycles received was 28 (range 19-61). *Results.* According to International Working Group 2006 criteria, 5 patients (23.8%) achieved complete response, 10 patients (47.6%) achieved partial response and 6 (28.6%) stable disease. Seventeen patients (80.95%) are still alive and continuing to receive AZA. Three patients (14.3%) died due to disease progression, one after 27, one after 29 and one after 33 months after the start of AZA. One patient died to infection disease (pneumonia). AZA treatment has lasted more than 3 years in 5 (23.8%) of the 21 patients. Peripheral cytopenias were the most common advent events for treatment. *Conclusions.* Allogeneic stem cell transplantation remains the only potentially curative treatment for MDS but the vast majority of patients with MDS are ineligible for transplantation because of comorbidities, advanced age, or lack of a suitable donor. Study in a larger series of patients is warranted, but our experience shows that in the absence of intolerable toxicity or disease progression, continued AZA treatment seems to improve the outcome of higher-risk MDS patients who are not eligible for transplantation.

PO-051

LA POSITIVITÀ DEL WT1 POST-INDUZIONE È UN IMPORTANTE FATTORE PROGNOSTICO PREDITTIVO DI RECIDIVA PRECOCE IN UNA CASISTICA DI 80 PAZIENTI AFFETTI DA LEUCEMIA ACUTA MIELOIDE

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Post consolidation minimal residual disease (MRD) evaluation represents a strong prognostic factor for AML outcome independent from cytogenetics, molecular markers and patients characteristics as hyperleukocytosis and age. Less data are available as regards the predictive value of MRD after induction, even thugh they are promising too. We evaluated post-induction and post-consolidation bone marrow mimimal residual disease (MRD) in 80 AML patients aged in median 57 years (range: 17-80) with 16 months median follow-up (range 2-55). We analysed abnormal leukemia immunophenotype (ALIP) by multiparameter flow cytometry (MPFC) and WT1 by RT-PCR as described by Buccisano *et al.* and Cilloni *et al.* Cytogenetic, NPM and FLT3 status were performed in 73, 63 and 78 patients respectively, defining the molecular cytogenetic risk in 62 patients. WT1 was +ve in 63/75 patients (85%) at diagnosis (median 1056; range:8.2-232,056), in 10/59 (16,9%) post induction (median 21; range:0,6-134.633) and in 8/50 (16%) post consolidation (median 18,6; range:0,8-45.338). MPFC MRD was +ve in 27/56 (48.2%) patients after induction and in 16/44 (36.4%) after consolidation. We analysed overall, 3 month, 6 month, 12 month Cumulative Incidence of Relapse (CIR) by MRD status (Table 1) and the predictive value of MRD adjusted by cytogenetic, NPM and FLT3 status, gender, secondary disease, hyperleukocytosis, age and kind of post consolidation treatment. 68 patients achieved CR, 27 patients relapsed in a median of 6.6 months (1-34 months) with a 45% 3 yr CIR: 12 patients relapsed at 3 months (CIR: 13%), 15 at 6 months (CIR: 18%), 23 at 12 months (CIR: 33%).

Table 1. Univariate analysis of CIR by MRD status.

	3 months Relapse Incidence (CIR)		6 months Relapse Incidence (CIR)		12 Months Relapse Incidence (CIR)		Relapse Incidence (3 yr CIR)	
		P		P		P		P
Post induction WT1		<0.001		<0.001		<0.001		<0.001
Positive	6/10 (60%)		6/10(60%)		9/10 (90%)		9/10 (90%)	
Negative	1/48 (2%)		4/48 (10%)		7/48 (19%)		10/48(44%)	
Post consolidation WT1		0.01		0.04		0.01		0.003
Positive	3/8 (44%)		3/8 (44%)		4/8 (62.5%)		5/8(100%)	
Negative	4/42 (10%)		6/42 (15%)		9/42 (23.5%)		11/39 (66.4%)	
Post induction MPFC		0.005		0.003		0.005		0.002
Positive	6/27 (25.5%)		8/27 (45.3%)		11/27 (51.5%)		12/27 (20.8%)	
Negative	0/29 (0)		1/29 (3.7%)		2/29 (8.3%)		4/29 (57.5%)	
Post consolidation MPFC		0.188		0.188		0.188		0.084
Positive	3/16 (22.7%)		3/16 (22.7%)		3/16 (22.7%)		6/15 (62.3%)	
Negative	2/28 (7.1%)		2/28 (7.1%)		2/28 (7.1%)		6/27 (26.4%)	

Patients receiving chemotherapy, Autologous and Allogeneic Transplant had 56%, 29% and 50% 3 yr CIR. We also observed a 3 yr CIR of 43% in NPM+FLT3- patients, compared to 39% in NPM-FLT3-, 60% in NPM+FLT3+ and 50% in NPM-FLT3+ respectively. Patients with WT1 +ve post induction and consolidation had a 3 month CIR respectively of 60% and 44% and a 12 month CIR of 90% and 62.5% respectively. Patients with MPFC +ve post induction and consolidation had a 3 month CIR of 25.5% and 22.7% respectively and a 12 month CIR of 51.5% and 22.7% respectively. Multivariate analysis identified post induction WT1 +ve status as the main predictor of 3 and 12 month CIR. Patients with WT1 +ve after induction had a 36 RR to relapse at 3 months (95% CI: 4.3-300; p=0.001) and a 14.3 RR to relapse at 12 months (95% CI: 4.7-43.9; p<0.0001) in comparison with WT1 -ve

patients (Figure 1). Hyperleukocytosis (WBC count >50,000/mcl at diagnosis) and age>60 yrs also significantly predicted 12 month CIR with a RR of 7.15 (95% CI:1.88-27.13; p=0.004) and 12.3 (2.75-55; p=0.001) respectively. In conclusion WT1 positivity post induction status confirmed its prognostic significance in our series targeting a subset of early relapsing patients with an extremely poor outcome.

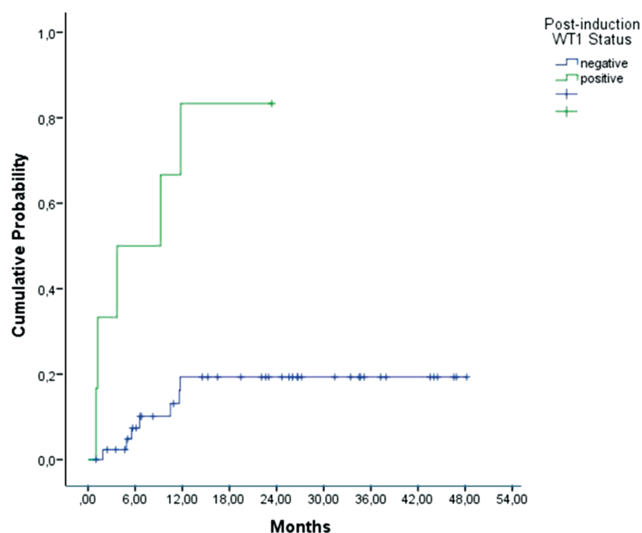

Figure 1.

PO-052

UNEXPECTED RAPID HEMATOLOGIC RESPONSE TO 5-AZACITIDINE (AZA) IN THERAPY RELATED MYELOID NEOPLASMS (t-MN)

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Introduction. t-MN are associated with unfavourable prognostic factors (poor performance status, cytopenias, chr 11, 5 or 7 abnormalities, complex karyotype). Median OS is less than 1 year even with intensive treatment. Demethylating agents, in particular AZA, demonstrated clinical activity in this setting. *Methods.* We retrospectively collected data of pts with t-MN and treated with AZA at our Institution in 2013. AZA was administered at the conventional dose of 75 mg/m2 daily for 7 days every 28 days (when possible). *Results.* 4 t-MN have been treated with AZA at our Institution in 2013. AZA was started at a median of 30 days from diagnosis. - A 83-year-old man, treated with radiotherapy for lung cancer in 2009 and 2012, was diagnosed with RAEB-1, complex karyotype (IPSS 2, int-2 risk) in August 2013. ECOG PS 1. He had 3 cytopenias. After the 1st AZA cycle, he obtained a profound PLT increase (PLT > 100x10^9/l). Concomitantly, despite fluorochinolone prophylaxis, he developed febrile neutropenia requiring admission to our Unit and treatment with iv antibiotics. This delayed AZA administration and the pt lost response obtained. After 55 days from beginning, cycle 2 was administered and complicated again by febrile neutropenia. During hospitalization disease progressed to AML and the pt died. - A 71-year-old man, previously treated for follicular NHL, developed AML (29% BM blasts, complex karyotype) in January 2013. ECOG PS 1. He had 3 cytopenias and started AZA in March 2013. After the 1st cycle we observed hematologic improvement (HI) on erythroid and platelet lines. HI was concomitant with febrile neutropenia which required hospitalization. The pt underwent 3 more cycles maintaining HI. Then, after a new sepsis, he lost response, and died. - A 67-year-old woman, in PR after treatment for NHL, started AZA after having been diagnosed with AML (29% BM blasts, 46,XX,del7(q22)[10]/46,XX,del6, del7[10]). ECOG PS 1. No HI was observed after 3 therapy cycles and infectious complications led to therapy stop. - A 80-year-old man was diagnosed with RAEB-2, karyotype 45,X,-Y,del5(q12q33)[13]/46,XY[7] (IPSS 2, int-2 risk) after chemotherapy for NHL. ECOG PS 1. He underwent 3 AZA cycles, with PLT and hemoglobin improvement soon after the 1st one, becoming transfusion independent. He died after the 3rd cycle for bowel perforation. *Conclusions.* Three pts out of 4 showed a rapid response to AZA. Time to HI was shorter than that described for *de novo* MN, possibly suggesting that AZA may act with mechanisms other than demethylation. Interestingly, in all cases HI occurred after the 1st AZA treatment, and in 2 cases it followed a severe infectious event. However response have been of short duration and the patients died of leukemic progression or septic complications. In t-MN pts AZA represents a therapeutic option that may induce rapid, albeit short, HI, and, in carefully selected cases, may prompt the investigation of late intensification.

PO-053

ACUTE PROMYELOCYTIC LEUKEMIA WITH RARE CASE PML exon 4 RARA exon 3 TRANSCRIPT: MRD MONITORING BY PATIENT SPECIFIC QUANTITATIVE RT-PCR ASSAY

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Over the last decade, the biomedical importance of RARα has been highlighted by its involvement in the pathogenesis of acute promyelocytic leukemia (APL). The chromosome translocation t(15;17), a hallmark for APL , disrupts the PML gene on chromosome 15 and the RARα gene on chromosome 17, resulting in chimeric PML-RARα and RARα-PML fusion genes. The breakpoints on chromosome 17 are consistently located in the second intron of the RARα gene, but on chromosome 15, there are different breakpoint cluster regions, located in intron 6, exon 6, and intron 3, as a result, three distinct types of PMLRARα transcripts, bcr1, bcr2 and bcr3 can be generated in different patients. The literature describes extremely rare variant transcripts in APL outside these cluster regions that concern different PML exons from those typical, for example exons 4 and 7. We describe a patient with the fusion transcript PML exon 4 and RARα exon 3 and the use of patient-specific TaqMan assay for monitoring of minimal residual disease (MRD). A 35-year-old woman patient was referred to our hospital in July 2013 with a clinical characteristics and morphological picture of bone marrow consistent with APL. Cytogenetic analysis using BM aspirates documented t(15:17)(q22;q21) in almost all the cell analyzed and FISH analysis using PML/RARalfa dual color dual fusion probe (Vysis) confirmed the presence of PML/RARalfa rearrangement Real-time quantitative PCR performed (according to EAC Protocols) on cDNA obtained from bone marrow at diagnosis detect a very low amount of bcr-3 PML-RARα transcript, this results was inconsistent with clinical data. Because of the discrepancy of cytogenetic findings and molecular results, PCR amplification of the PML-RARα gene were performed using Biomed-1 protocol. An larger size of PML-RARα amplicon was detected and sequenced and the exact fusion transcript was identified. The fusion transcript consist of the end of exon 4 in PML and exon 3 of RARα gene. For MRD study we performed quantitative real-time PCR by a modified EAC protocol. We developed a patient specific quantitative MRD assay, briefly: we designed and utilized a forward primer in PML exon 4 and the same reverse primer and probe of EAC protocol. The system did not amplify other related heterogeneous c-DNA sample and the assay achieved sensitivity of 5 log.. The patient achieved complete remission after induction therapy and remained in molecular remission by RT-PCR, at 3, 6 and 9 months of follow-up. In the last ten year in our centre we observed 124 new diagnosis of APL and only the case described showed atypical transcript . In this case, in agreement with the few cases described in the literature, seems that APL with PML exon 4-RARα exon 3 fusion transcript never show a poor prognosis. Patient-specific MRD assay is a useful and necessary method to obtain a correct monitoring MRD in APL rare patient with variant fusion transcript.

PO-054

CLEC12A: A NEW AML STEM CELL-ASSOCIATED ANTIGEN

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Introduction. Acute myeloid leukemias (AML), a heterogeneous and complex group of diseases, is a clonal malignant disorder derived from a small number of leukemic stem cells (LSCs), which are sustained by self-renewing and responsible for the propagation of leukemic blasts (LBs). Monoclonal antibodies have emerged as effective targeted therapies for the treatment of human malignancies and their mechanisms of action are able to deliver the therapeutic effects with minimal toxicity. The aim is the identification of cell surface antigens which could be preferentially expressed on AML LSC compared with normal hematopoietics stem cells and that could be helpful to target therapies. *Methods.* On 16 AML patients (pts) at diagnosis (peripheral blood of 12 pts and bone marrow of 4 pts) myeloblast leukemic cells were identified using a FACSCanto flow cytometer (Becton Dickinson), based on low expression of CD45 and low side scatter (SSC) properties (CD45 low/SSC low). On these subpopulation we used an antibody panel against cell surface antigens, for the detection of immature markers and potential leukemia-associated antigens: CD34, CD38, CD90, CLEC12A (C-type lectin domain family 12 member A), CD44, CD99, TIM-3 (T cell immunoglobulin mucin-3), CD32, CD133, CD74, CD47, CD58, CD25, CD22, CD96. *Results.*. The proportion of LBs was positively correlated with CD45 low/SSC low (median 51,8 %). This gated population of LBs were positive for CD34 (median 44,65%), CD38 (median 18,75%), CD90 (median 0,90%), CLEC12A (median 93,7%), CD44 (median 99,9%), CD99 (median 96,6%), TIM-3 (median 84,4%), CD32 (median 14%), CD123 (29,15%), CD133 (median (8,95%), CD58 (median 97,5%), CD47 (median 99,9%), CD74 (median 3,2%), CD25 (median 0,6%), CD96 (median 87%), CD22 (median 0,55%). *Conclusions.* The expression of CD34 and CD38 antigens is heterogeneous in LBs. In particular we found that the 50% of patients were CD34+ and 50% were CD38+. CLEC12A, CD44, CD99, TIM-3, CD58, CD47 and CD96 were highly expresses in LCSs. CD90, CD32, CD123, CD133,CD74, CD25 and CD22 were low. Interestingly we identified that the expression of CLEC12A distinguished two different populations: the CLEC12Ahigh cells correlated with the blast cells CD45low/SSClow; on the other hand, CLEC12Alow cells could be compared with CD45high/SSClow population, representing normal hematopoietic cells. *Conclusions*. This marker is a good candidate to target therapies against leukemic stem cells. However further studies with an higher number of patients must be carried out to confirm that CLEC12A is an appropriate antigen for a new monoclonal antibody-based therapy.

PO-055

AMERICAN GINSENG (PANAX QUINQUEFOLIUS) SUPPLEMENTATION IMPROVES FATIGUE IN MYELODYSPLASTIC SYNDROMES: A CLINICAL AND BIOLOGICAL STUDY

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Introduction. Myelodisplastic Syndrome (MDS) is a clonal hematopoietic stem cell disease characterized by cytopenia, multilineage dysplasia, ineffective hematopoiesis, and increased risk of development of acute myeloid leukemia. MDS is associated with Chronic Fatigue Syndrome (CFS) that can reduce patient's quality of life (QoL). It has been proposed that Oxidative stress (OS), an unbalance between Reactive Oxygen Species (ROS) and Total Antioxidant Capacity (TAC), is a possible link between MDS and CFS. American ginseng (Panax quinquefolius) showed antioxidant effect by scavenging free radicals and activating Superoxide Dismutase (SOD) which enhances the normal dismutation of superoxide, one of the strongest oxidant, forming hydrogen peroxide. *Methods.* Eleven MDS patients (1F/10M, age range 68-83 years) were recruited at our Hospital; CFS was diagnosed by using Edmonton Symptom Assessment System Scale (ESAS Scale). All participants gave their informed consent. Serum ROS and TAC levels , assessed by using a spectrophotometric method (dROMs test and OXY-Adsorbent test; Diacron International Grosseto; Italy, respectively) and CFS were evaluated at baseline (T0) and after 2-month EUQUINAX™ supplementation (T1). T0-T1 comparison was conducted by Mann-Whitney-Wilcoxon test (MedCalc software) and significance level was settled at $p<0.05$* (T0 *vs* T1). *Results.* Oxidative stress results are reported in the Table 1 as median (InterQuartile Range, * statistically significant). At baseline, even if ROS concentrations were below the cut-off value in the most of cases, a significant direct correlation ($p=0.04$; $r=0.61$) was found between ROS levels and the ESAS Scale total score. Most of the patients showed TAC under the cut-off value. TAC levels decreased significantly ($p=0.03$) at T1 and a positive correlation ($p=0.09$) was found between the percentage of ROS' decrease and the percentage of ESAS Scale total score's decrease. Finally, we observed a significant ESAS Scale total score improvement at the end of the supplementation. *Conclusions.* Our preliminary results show a significant reduction of CFS evaluated with ESAS scale after 2-month of American ginseng (EUQUINAX™) treatment. This clinical effect is accompanied by a decrease of TAC, probably due to American ginseng ability to buffer hydrogen peroxide excess, representing a coadjuvant agent in ROS detoxification.

Table 1.

Analyte (*cut-off* value)	T0	T1	percentage of decrease or increase (%)
ROS **(<300 U/Carr)**	**248.0 (217.0;297.0)**	**244 (206.7;270.7)**	**0.7 (range -8;10)**
TAC **(>350 µmolHClO/mL)**	**336.0 (245.2;345.7)**	**241.0 (201.0;286.5)***	**-20 (range -40.3;-3.6)**
ESAS Scale total score	**31 (11;38)**	**12 (6;22)***	**-52.5 (range -92; -12)**

Leukemias and Myelodysplasias II

PO-056

INDOLEAMINE 2,3-DIOXYGENASE (IDO) IS ASSOCIATED WITH HIGH INCIDENCE OF CHEMOREFRACTORY DISEASE IN ACUTE MYELOID LEUKEMIA (AML) PATIENTS

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IDO is a heme-containing enzyme that catalyzes the rate-limiting step in tryptophan degradation; it is able to inhibit T-cell function by inducing the transformation of T-cells into regulatory T-cells. IDO is involved in immune tolerance induction during pregnancy, infection, transplantation, autoimmune diseases and neoplasias, including acute myeloid leukemia (AML), where it is expressed in a significant number of patients. Here, we addressed the correlation between IDO expression by AML cells, risk factors at diagnosis and patients' outcome. Adult AML patients from the Hematology Institute "L. and A. Seràgnoli" in Bologna were analyzed for risk characteristics at diagnosis and IDO expression by RT-PCR and Western-Blot analysis. Patients were stratified according to age at diagnosis, *de novo* or secondary disease, leucocytosis, cytogenetics, FLT3 and NPM mutational status. Fifty-two AML patients were analyzed for IDO expression both at gene and protein level. According to IDO transcript levels, patients were divided into IDO-negative (21%) and IDO positive (79%). Positive patients were divided into three subgroups according to protein level: IDO-low (78%), IDO-intermediate (10%) and IDO-high expression (12%) patients. No statistically significant differences in the recurrence of prognostic characteristics at diagnosis between the four groups were observed, even though IDO-negative and IDO-low expression patients showed a higher median age at diagnosis than IDO-intermediate and IDO-high expression patients and an increased frequency of high-risk cytogenetics was found in IDO-high expression patients. Response to induction chemotherapy regimen was then analyzed: only patients who received cytotoxic chemotherapy were evaluated for response. Refractory patients were 60% among those who express IDO at high level and 27% among IDO-negative patients. A logistic regression analysis showed a significant difference between IDO-negative and IDO-high expression patients in terms of complete remission rate: IDO-high expressing patients show an increased proportion of refractory disease than IDO negative patients. This finding may suggest a correlation between chemosensitivity and the induction of anti-leukemia immune response (immunogenic cell death). Since chemotherapy is known to kill tumor cells in an immunogenic manner, we hypothesize that IDO expression in AML cells may contrast such process, thus resulting in a decreased activation of leukemia-specific T cells.

PO-057

GENOMIC ANALYSIS OF NOTCH MUTATIONS IN A CASE OF ALAGILLE SYNDROME WITH ACUTE LYMPHOBLASTIC LEUKEMIA

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Introduction. Alagille syndrome (ALGS), or arteriohepatic dysplasia, is a congenital multisystem disease due to Notch signalling pathway mutations, most commonly affecting JAG1 (ALGS type 1), and more rarely NOTCH2 (ALGS type 2), leading to hepatic, lung, renal and ocular dysfunction (chronic cholestasis, peripheral pulmonary artery stenosis, dysplastic kidneys pigmentary retinopathy), and skeletal abnormalies (minor vertebral segmentation, characteristic facies, posterior embryotoxon/anterior segment defects). ALGS is an autosomal dominant disease, but it is characterized also by variable penetrance and clinical expression and somatic/germline mosaicism. *Methods.* A 20-year-old man with ALGS was admitted to the University Hospital of Verona because of pancytopenia. Following analyses led to the diagnosis of Philadelphia chromosome/bcr-abl-negative, CD10-positive, B-lineage acute lymphoblastic leukemia (common B-ALL). *Results.* In order to identify the genetic components involved in this complex phenotype, we sequenced the exome of a bone marrow sample collected from the patient. By genome interpretation with Knome pipeline applied to the reference genome UCSC hg19, we found missense variants both in NOTCH2 (E38K) and JAG1 (P871R) genes that are mainly involved in the syndrome, although their effect on protein function was predicted not to be deleterious. However, we detected putative damaging mutations in genes such as PAX5 (R38H) and NOTCH1 (K1821N) which might be strongly related to the observed disease. In fact, PAX5 is a member of PAX protein family of transcription factors implicated into regulation of early development, that binds NOTCH2 and likely altering its functionality. On the other hand, NOTCH1 is involved in cell growth and proliferation and thus the predicted alteration of function of the corresponding protein may have an important role in neoplastic transformation. *Conclusions.* Overall, this study provides novel insights in the pathogenetic development of B-ALL in a patient with dysregulated Notch signalling due to ALGS. On the basis of the genetic abnormalities found and here discussed, we suggest a model that may explain the molecular link among the different signalling pathways involved in the development and relapse of B-ALL.

PO-058

ROLE OF STROMAL CELL-MEDIATED NOTCH SIGNALING IN AML SURVIVAL AND RESISTANCE TO CHEMOTHERAPY

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Introduction. Our group has recently shown that bone marrow-mesenchymal stromal cell (BM-MSCs)-mediated Notch signaling may control survival and chemoresistance of B-acute lymphoblastic leukemia (B-ALL) and chronic lymphocytic leukemia (CLL) cells. Conversely, the role of Notch signaling in acute myeloid leukemia (AML) remains controversial, as its contribution to the crosstalk between BM-MSCs and leukemic cells is still unknown. Thus, we evaluated the role of the Notch pathway in the proliferation, adhesion, survival and chemoresistance of different AML cell lines (HL-60, THP1, U937) and AML primary cells in co-culture with BM-MSCs. *Methods.* AML cell lines were obtained from ATCC, while AML primary cells were obtained after informed consent from bone marrow samples of 30 AML patients, according to the Institutional guidelines. PCR, FACS analysis and western immunoblotting were used to study the expression of Notch receptors and ligands, as well as Notch activation, in AML cells and BM-MSCs. AML cells were co-cultured with BM-MSCs at 10:1 and 1:1 (AML:BM-MSCs) ratios for 2 to 3 days in presence of Cytarabine, Etoposide, Idarubicin, as well as in presence or absence of anti-Notch-1, -2, -3 blocking antibodies or Notch pathway gamma secretase inhibitor-XII (GSI-XII). Cell viability was evaluated by Annexin-V/PI and MTT assay; proliferation and cell cycle were assessed through CFSE dilution and Propidium Iodide methods, respectively. *Results.* Both AML cell lines and primary AML cells expressed Notch receptors and ligands, with a constitutive basal activation of the Notch system. The expression and activation pattern was modulated after 3 days of co-culture with BM-MSCs. The blockade of Notch signaling by GSI-XII was capable of inhibiting AML cell proliferation and adhesion to BM-MSCs. The addition of chemotherapeutic agents decreased AML cell viability in culture, while a significant rescue from apoptosis was observed when cocultured with BM-MSCs. Notch signaling blockade by GSI-XII in presence of chemotherapeutic agents significantly lowered the supportive role of BM-MSCs towards AML cell lines. Consistently, the addition of anti-Notch-1 or a combination of anti-Notch-1,-2,-3 blocking antibodies rescue the chemosensitivity of primary AML cells in co-culture with BM-MSCs. *Conclusions.* Notch signaling plays a role in the proliferation, adhesion and chemosensitivity of AML cells and partially reverts their BM-MSC-induced chemoresistance.

PO-059

MODULATION OF GLYCOLYTIC METABOLISM BY DICHLOROACETATE INDUCES APOPTOSIS IN ACUTE MYELOID LEUKEMIA CELLS

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Introduction. Dichloacetate is a small pyruvate-mimetic molecule that modulates cellular metabolism, inhibiting lactate production and diverting pyruvate into oxidative phosphorylation. Recent evidences state that leukemia cells rely on high glycolytic fluxes in order to generate the ATP needed for proliferation, a metabolic switch mediated by oncogene-driven signalling perturbations. This peculiar metabolism may represent a promising target for therapeutic intervention. Thus we investigated the effects of metabolic reprogramming by DCA exposure in acute myeloid leukemia (AML) cell lines and primary samples. *Methods.* Effects of DCA exposure on 5 human AML cell lines (U937, OCI-AML3, OCI-AML2, HL-60, MOLM13), on 12 AML primary samples and on normal peripheral blood lymphocytes (NPBLs) were evaluated by cell counts, apoptosis induction (AnnV) and changes in glucose and lactate culture medium levels (GEM4000, Instrumentation Laboratory, UK). Glucose consumption rate (GCR) and lactate production rate (LPR) were calculated according to Li *et al.* (Biotechnol. Appl. Biochem., 2005). Real-time lactate production rates (ECAR) and oxygen consumption rates (OCR) were measured by a Seahorse XF24 Analyzer (Seahorse Bioscience, MA, USA). *Results.* All AML cell lines are characterized by a higher glucose catabolism when compared to normal and activated NPBLs: at 24h the U937 cell line had a 6.7 fold higher GCR than activated NPBLs. Real time metabolic analysis in the same cell line showed that DCA injection (5mM) induced a temporary drop in lactate production (36.5%, $p=0.0014$), while control condition remained unchanged; ECAR returned to values equivalent to those of control by 30 minutes of DCA exposure. During this time interval (30 min), the oxygen consumption gradually rose by 45% compared to control ($p=0.014$), remaining stable in the following time-points. Longer DCA exposure times (1-7,5mM) induced a dose- and time-dependent cell growth arrest and apoptosis on U937, MOLM13, OCI-AML3 (72h IC50=2.62, 5.1, 5.81mM, respectively). Conversely, HL60 and OCI-AML2 proved resistant (IC50=nd). A pro-apoptotic activity was observed at 72h of DCA *in vitro* exposure on all primary samples: AnnV positive cells ranged from 19.3±9.5% (vehicle) to 31.4%±14,8% (1mM, $p<0.001$), 43.3±18,7% (2,5mM, $p<0.001$), 56.7±26.2% (5mM, $p<0.001$), 66.1±20.2% (7,5mM, $p<0.001$). No significant effects were observed on both normal and activated NPBLs. *Conclusions.* DCA is effective in inducing a metabolic shift in leukemia cells, increasing oxidative phosphorylation levels. This effect is associated with apoptosis induction in primary AML samples. Further studies are needed to elucidate the mechanism of DCA apoptosis induction.

PO-060

A COMBINATION OF BORTEZOMIB WITH CX-4945, A CASEIN KINASE 2 (CK2) INHIBITOR, HAS SYNERGISTIC CYTOTOXIC EFFECTS IN ACUTE LYMPHOBLASTIC LEUKEMIA (ALL) CELL LINES

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Introduction. The proteasome inhibitor bortezomib is a new treatment option for patients with refractory or relapsed ALL, particularly when used in combination with conventional chemotherapy or other targeted agents. Indeed, a limited efficacy of bortezomib alone in ALL patients has been reported. A terminal pro-apoptotic endoplasmic reticulum (ER) stress/unfolded protein response (UPR) is one of the several mechanisms of bortezomib-induced apoptosis. CX-4945, a potent CK2 inhibitor, has been found to induce apoptotic cell death in T-ALL preclinical models, via perturbation of ER/UPR pathway. Here, we have explored the cytotoxic effects of a bortezomib/CX-4945 combination in a panel of B- and T-ALL cell lines. *Methods*. B- (KOPN-8, NALM-6, RS4;11) and T- (MOLT-4, Jurkat, CEM-R) ALL cell lines were pretreated with bortezomib (Selleck Chemicals, Houston TX, USA), for six hours and then with CX-4945 (Selleck Chemicals, Houston TX, USA), for 16/24 hours. MTT assays were performed to analyze cell viability. Apoptosis induction was evaluated by Annexin V/PI staining and flow cytometric analysis. Protein expression was studied by western blot. *Results*. Cells were cultured in the presence of bortezomib or CX-4945, either alone or in combination at a fixed ratio. The combined treatment was more effective in reducing cell viability in all B-ALL cell lines and in MOLT-4 cells. Annexin V/PI staining was performed; interestingly, no synergism was detected if the two drugs were administered together since the beginning of treatment. In response to treatment with 2.5 nM bortezomib followed by 5 μM CX-4945, we detected an increase in apoptosis in B-ALL cell lines and in MOLT-4 cells after 24 h. Western blot analysis for the cleaved forms of caspase-3 and PARP, confirmed a higher apoptosis induction by the combined treatment. A reduction in anti-apoptotic Bcl2 concomitant with an increase in pro-apoptotic Bax, suggested that bortezomib/CX-4945 treatment caused a mitochondrial apoptosis. IRE1a and CHOP (established markers of ER stress/UPR-mediated apoptosis) levels increased in response to the combined treatment, in contrast the expression of GRP78/BIP (a marker of UPR activation) decreased, suggesting that a potential mechanism by which the drug combination induced cell death, involved ER stress induction by bortezomib and the inability to respond by adequate activation of the UPR signaling which was blocked by CX-4945. *Conclusions*. Here, we demonstrated that the proteasome inhibitor bortezomib and the CK2 inhibitor CX-4945 interact in a synergistic manner to induce apoptosis both in B- and in T-ALL cell lines. Drug cytotoxicity was associated with modulation of the ER stress/UPR signaling pathway. Importantly, the synergism was observed only when bortezomib treatment preceded CX-4945 administration. Therefore, our findings support clinical application of bortezomib in combination with CX-4945 in B- and T-ALL treatment.

PO-061

NON-CANONICAL NOTCH SIGNALING IN T-ALL: A PHOSPHO-FLOW CYTOMETRY STUDY

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Introduction. Deregulated Notch1 signaling has a fundamental role in the pathogenesis of T cell acute lymphoblastic leukemia (T-ALL), in which the majority of human and murine tumors have acquired activating mutations that lead to aberrant increases in Notch1 signaling. NOTCH1 encodes a transmembrane receptor acting as a ligand-activated transcription factor and playing an important role in cell differentiation, proliferation, and apoptosis. Notch1 signaling initiates when the ligand, from either the Jagged or Delta families, binds to the receptor and induces successive proteolytic cleavages, resulting in the release and nuclear translocation of the Notch1 intra-cellular domain (NICD). In the nucleus, NICD assembles a transcriptional complex, leading to de-repression/activation of specific target genes. Besides this "canonical" signaling pathway involving ligand-induced cleavage of Notch for transcriptional regulation, a ligand- or transcription-independent (non-canonical) function of Notch has also been reported in various systems across species. However, in most cases, the key mediators of non-canonical Notch signals are unclear, and the proposed mechanisms appear to vary with context. In T-ALL, Notch non-canonical signals and their possible cytoplasmic mediators have been not investigated so far. *Methods.* To study the non-canonical Notch signaling pathway in T-ALL, we used multi-parametric phospho-flow cytometry to simultaneously determine protein expression and protein post-translational modifications (*i.e.* phosphorylation) at a single cell level in T-ALL cell lines stimulated by the Notch1 ligand Jagged1. Then, protein expression of specific Notch target genes, as readout of Notch1 signaling activation, has been analyzed at slower time points whilst phosphorylation of signaling proteins, which are crucial for T-ALL pathogenesis, has been measured at rapid time points, to identify possible cytoplasmic mediators of the non-canonical Notch1 signaling. *Results.* We showed that, besides the expression of specific Notch target proteins, Notch1 activation induced the rapid phosphorylation of cytoplasmic proteins, namely STAT3, Akt, and Rb. More-

over, Notch1 activation evoked heterogeneous signaling profiles across different cell lines, which recapitulate ontogenetic stages in T-ALL. *Conclusions.* This study showed that "non canonical" cytoplasmic signals are induced in T-ALL in addition to slower transcriptional responses traditionally attributed to Notch activation. In addition, multi-parametric phospho-flow cytometry enables to distinguish Notch signaling network profiles that are associated with the T-ALL ontogeny stages. Identifying cytoplasmic mediators of the Notch non-canonical pathway as biologically relevant signaling hubs may represent new challenges for anti-Notch1 therapy in T-ALL.

PO-062

NOT PUBLISHED

PO-063

ASSESSMENT OF THE EFFECT OF SPHINGOSINE KINASE INHIBITORS ON APOPTOSIS, UNFOLDED PROTEIN RESPONSE AND AUTOPHAGY OF T-ACUTE LYMPHOBLASTIC LEUKEMIA CELLS: INDICATIONS FOR NOVEL THERAPEUTICS

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Introduction. Sphingosine 1-phosphate (S1P) is involved in many processes such as cell survival, growth, migration, and cancer. S1P is formed by the phosphorylation of sphingosine by sphingosine kinase 1 (SK1) or sphingosine kinase 2 (SK2). While the role of SK1 in cancer is well established, the role of SK2 in regulating apoptosis is still a matter of debate, even though emerging evidence has highlighted the importance of SK2 in cancer. Therefore, SKs represent a promising target for cancer therapy. Recently, the importance of S1P in hematological malignancies has been described. Here, we analyze the therapeutic effects of 2-(p-Hydroxyanilino)-4-(p-chlorophenyl)thiazole (SKi), an SK1/2 inhibitor, and (R)-FTY720 methyl ether (ROMe), a SK2-selective inhibitor, in T-ALL cell lines. *Methods*. T- ALL cell lines (Molt-4, Jurkat, CEM-R) were treated with SKi and ROMe for 40 h, then MTT assays were performed to analyze cell viability. Apoptosis induction was evaluated by Annexin V/PI staining. Protein expression was studied by western blot (WB). *Results*. SKi and ROMe induced a decrease in cell viability, as demonstrated by MTT assays. Annexin-V/PI staining and flow cytometric analysis of cells treated with a SKi concentration equivalent to the IC50 documented the occurrence of apoptotic cell death. This was confirmed by WB analysis for caspase and PARP cleavage in all the cell lines. However, we also observed a SKi-induced autophagy by means of WB and transmission electron microscopy analysis in Jurkat and CEM-R cells. We then analyzed the expression of ER stress/UPR hallmarks. SKi activated the ER stress/UPR pathway and this occurred following apoptosis and correlated with autophagy. The combination of SKi and chloroquine (an autophagy inhibitor) induced a decrease in cell viability, indicating that the UPR/autophagic response is likely to be a protective mechanism. In Molt-4 cells, we detected an increase in SK1 expression after 40 h of treatment with SKi, that could represent an attempt of cells to escape SKi-induced apoptosis, as high SK1 expression is known to enhance cell growth and survival. Notably, SKi synergized with vincristine at concentrations of SKi that were much below its respective IC50, suggesting that vincristine sensitized T-ALL cells to SKi and that combining chemotherapeutic agents with SK inhibitors could be feasible in the treatment of T-ALL. Finally, ROMe treatment induced an autophagic type of cell death. *Conclusions*. We report herein that SKi and ROMe affect T-ALL cell viability, but they exert their effects through different mechanisms. While ROMe induces an autophagic cell death, SKi induces apoptosis. Moreover, for the first time, we demonstrated that SKi activates an ER stress/UPR pathway in T-ALL cells and this is linked with a protective autophagic response. Thus, our findings indicate that SK1 or SK2 may represent potential targets for treating T-ALL, thereby enabling better management of this cancer.

PO-064

UNCX: A NOVEL HOMEOBOX GENE IN ACUTE MYELOID LEUKEMIA

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Introduction. Homeobox (HB) genes play a pivotal role in normal and malignant hematopoiesis and are frequently involved in the development of AML. Recently, we identified a AML M5 case with a t(7;10)(p22;p14) as the sole cytogenetic abnormality, resulting in the ectopic expression of the HB gene UNCX (7p) due to a position effect. Interestingly, two ESTs mapping in intron II are reported in UCSC Genome Browser, suggesting the possible occurrence of alternative transcripts. We thus investigated the UNCX expression pattern in a cohort of AML cases, in a large panel of AML and cancer cell lines, none of them carrying the t(7;10) translocation. *Methods.* FISH experiments and SNP Array CGH were performed to characterize the translocation and possible unbalanced alterations, respectively. RT-qPCR and RT-PCR analyses were carried out to quantify UNCX expression level, and to detect alternative transcript isoforms, respectively. An Exon Array Expression assay was performed to identify differences in the gene expression profile due to UNCX activation. The data were analyzed by the GeneGo software and then validated by RT-qPCR. As UNCX is located within a large CpG island, its methylation status was evaluated by means of Mass Array Sequenom. *Results*. UNCX ectopic activation was detected at different levels in 75,4% (46/61) of the AML patients and in 14.7% (11/75) of the investigated AML cell lines, regardless of the FAB subtype. The methylation analysis of the CpG island at the UNCX locus did not reveal any difference between the translocated case and other AML controls, disclosing a general hypomethylated status. Moreover, we found additional transcript variants, including unspliced portions of UNCX intron II. In silico translation of these isoforms revealed the partial loss of the UNCX homeodomain. Interestingly, we found a clearly different gene expression profile between UNCX positive and negative samples. The GeneGo enrichment analysis showed that most of the differentially expressed genes were involved in hemopoiesis, cytoskeleton rearrangement and cell proliferation. *Conclusions*. We describe the ectopic expression of UNCX in AML, never reported in human cancer, so far. Notably, we found the activation of this HB gene in one patient due to a position effect, as well as in a large group of additional AML cases and tumor cell lines, although not showing the t(7;10) translocation. Since we found no difference in the methylation status of the gene promoter region in both positive and negative patients, the mechanism behind UNCX ectopic expression and its role in leukemogenesis are still to be elucidated. Moreover, the role of the identified alternative UNCX transcript variants needs to be clarified. Our results suggest a role of UNCX in leukemic cellular proliferation. To validate this hypothesis, we are performing retroviral-mediated UNCX gene transfer into cord blood CD34+ cells. The results are presently under evaluation and validation.

PO-065

CHANGE OF IMMUNOPHENOTYPE AND MOLECULAR CHARACTERISTICS AT RELAPSE IN AML: A CASE REPORT

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In Acute Myeloid Leukemia (AML) relapse may represent a clonal evolution of the disease or it may be the occurrence of treatment-related secondary AML. In this study we report a peculiar case of AML patient that relapsed after 2 year from diagnosis. On March 2012 a diagnosis of AML M5a was performed, according to the FAB criteria and the WHO classification, in a 66 years old man admitted to our hospital for

leukocytosis, thrombocytopenia, fever and respiratory failure. Initial laboratory investigations showed: white cell count 51,3x109/L, hemoglobin concentration 88g/L, platelet count 25x109/L. Examination of blood film showed a 50% of myeloid blast cells. Diagnosis of AML was confirmed by morphology of bone marrow aspirate. Flow cytometric immunophenotyping, conventional cytogenetic test, FISH and molecular analysis were also performed on bone marrow aspirate to further characterized AML prognostic risk. Immunophenotypic studies performed on the bone marrow aspirate showed that leukemic blast cells were characterized by CD45low, CD33, CD15, CD64, CD56, CD38, HLADR, MPO7 expression while CD14 and CD13 were expressed on only a minority of cells; blast cells did not express CD34 antigen. Conventional cytogenetic analysis showed a normal karyotype, and FISH analysis using different AML specific probe did not detect aberrations typical for AML. Molecular analysis for NPM1, FLT3 and WT1 showed NPM1 mutated, FLT3 wild type and low level copy number of WT1. Based on these findings, patient was classified as low risk and he was treated with 4 cycles of conventional chemotherapy (inclusive of HDAC) with achievement of complete remission (CR) after induction therapy. Autologous stem cell transplantation was not performed because he failed autologous peripheral stem cell collection. The patient maintained CR until April 2014 (2 years after last chemotherapy), when leukopenia (2.0 x109/L, severe anemia (89g/L) and thrombocytopenia (22 x109/L) were recorded and AML relapse was confirmed. Bone marrow immunophenotypic studies showed leukemic cells (20%) with a different immunophenotypic pattern compared with that recorded at the onset. In fact leukemic cells expressed CD45low, CD34, CD13, CD117, HLADR, CD123, MPO7; while CD33 and CD38 were expressed on only a minority of cells. Cytogenetic and FISH were confirmed negative while molecular tests showed a loss of NPM1 mutation, and a significantly increased of WT1 copy number, while the FLT3 mutation was retained wild type. Our data displayed that leukemic population at relapse was characterized by immunophenotypic and molecular features completely different to leukemic population at onset suggesting strongly that relapse was due to the occurrence of treatment-related secondary AML.

PO-066

APPLICATION OF THE NEWER LABORATORY PROGNOSTIC INDICATORS ON NEW DIAGNOSIS OF YOUNG AML PATIENTS OF THE LAST THREE YEARS IN AREA VASTA ROMAGNA: ANALYSIS OF DISTRIBUTION OF CYTOGENETIC ABNORMALITIES AND CONSIDERATIONS ABOUT THE INTERMEDIATE RISK GROUP WITH +8 POLISOMY

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Introduction. The integration of pretreatment parameters, including cytogenetics and evaluation of molecular markers, represents a challenge in the diagnostic algorithms of younger patients (< 60 years) diagnosed with *de novo* AML, for the implications in the post-remission therapy choice. Diagnostic karyotype is one of the most powerful independent prognostic indicators, with three cytogenetic risk groups identified by the MRC cytogenetic classification system (Blood 1998) and recently refined on a study cohort of 5876 AML cases. The role of an extended molecular analysis on AML at diagnosis is still controversial, even if the impact of the mutational status of NPM1 genes and FLT3-ITD on outcome in the intermediate cohort of patients is established today, and the European Leukemia Net in 2010 entered the normal karyotype (NK) with NPM1 positivity and FLT3 wild type in the low-risk group. We retrospectively analyzed the 36 AML cases of AVR of the last three years with successful karyotype analysis in spite to stratify our patients at diagnosis according to MRC and ELN recommendations. We also performed a correlation with prognosis in the restricted group of patients with +8 alterations, for which the prognostic value remain uncertain in larger studies. *Methods.* Cytogenetic analysis was performed on 20 metaphases from BM aspirates using standard procedures. Analysis of less than 10 metaphases was regarded as a failure. For molecular analysis DNA was obtained from mononuclear cells of BM; the presence of exon 12 mutations in NPM1 gene was tested by sequence analysis; the presence of FLT3 ITD was performed using 'FLT3 Mutation Assay – Gel Detection' (InVivoScribe Technologies). *Results.* The median age of the patients was 52 years (range, 18-59 years). Overall 16 of 36 (44,4%) of patients had a NK; frequencies of the various cytogenetic abnormalities identified are shown in Table 1.

Table 1.

	Patients, no. [%] (MRC frequencies*)	Cytogenetic RISK
NORMAL KARYOTYPE	16/36 [44,4%]41%	**intermediate**
RECURRENT BALANCED ABNORMALITIES	9/36 [25%] (28%)	
t(15;17)	5/36 [13,8%] (13%)	**good**
t(8;21)	1/36[2,8%] (7%)	**good**
inv(16)	1/36[2,8%] (5%)	**good**
t(6;9)	1/36[2,8%] (1%)	**adverse**
inv(3)	1/36[2,8%] (1%)	**adverse**
MDS –RELATED ABNORMALITIES	5/36 [13,8%](18%)	**adverse**
45,XY,der(2),der(7)t(7;17)(q22;q11), -14,der(17) [20]	1/36[2,8%]	
45,XX,-7 [10]/ 46,XX,del(7)(q22)[8]/47,XX,+21 [2]/ 46,XX [1]	1/36[2,8%]	
45,XX,del(7)(q31),-18 [10]/ 46,XX,del(7)(q11),-18,+mar [5]/ 46,XX [9]	1/36[2,8%]	
45~47,XX,-3,-5,-13, der idup(17),- 18,-20,+21,+ 3~5mar [13]/46,XX [7]	1/36[2,8%]	
50,XX,+8,del(11)(q23),+3dmin [20]	1/36[2,8%]	
OTHER ABNORMALITIES		
+8	4/36 [11,1%] (10%)	**intermediate**
del(3)	1/36 [2,8%] (2%)	**adverse**
+der(1)t(1;14)(p11;q11)	1/36 [2,8%] (2%)	**intermediate**

* Grimwade D, Hills RK, Moorman AV, Walker H, Chatters S, Goldstone AH, Wheatley K, Harrison CJ, Burnett AK; National Cancer Research Institute Adult Leukaemia Working Group. Refinement of cytogenetic classification in acute myeloid leukemia: determination of prognostic significance of rare recurring chromosomal abnormalities among 5876 younger adult patients treated in the United Kingdom Medical Research Council trials. Blood. 2010 Jul 22;116(3):354-65.

Analysis for cytogenetic-risk group assigned 7 of 36 patients to the low risk group (19.4%), 21 patients to the intermediate risk group (INT, 58%) and 8 to the adverse risk group (22%). The application of molecular analysis on the group with NK (=15 evaluable pt) led to reassignment of a further 4 cases to low- risk group. The most common alteration in the INT (Table 2) excluding NK (n=16) was represented by alteration of +8 (n=4), and they showed a dismal prognosis, with a median OS of 3.5 months (range, 2-14). The only patient with favorable mutational status (NPM1+/FLT3ITD-) had +8 pentasomy with a mutation of NPM1 (type Gm).

Table 2.

	Age	Diagnostic karyotype	Mutational Status	WBC	FAB	Flow cytometry analysis	Response	Relapse	DFS	OS
1	36	49,XY,+8,+8, +8 [21]/46,XY[1]	NPM1 mut Gm; FLT3 non mut	18010	M5	CD4+,CD11b+,CD11c+, CD14--/+,CD15+,CD16--/+,CD33+,CD38+, CD56+,CD58+,CD64+,HLA-DR+	CR	early relapse	1 month	14 months
2	54	47,XX,+8 [20]	NPM1 mut A; FLT3 ITD mut	28600	M2	CD34+ eter CD33+ bright CD11b+ eter CD13+ dim CD117+/- HLADR+ eter MPO+ dim.	RES	RES	0	3 months
3	54	47,XY,+8[20]	NPM1 WT; FLT3 WT	1850	M5	CD4+,CD11b+,CD13+,CD15+,CD33+;CD38+,CD56+,CD64+,CD71+,CD117+,HLA-DR+, MPO+	CR	NO	5 days	1 month
4	50	47,XY,+8 [4]/ 46,XY [18]	NPM1 WT; FLT3 WT	125000	M5b	CD4+, CD56-/+, HLA-DR+ , CD33+ , CD14-/+, CD64+, CD11b+ , CD11c+ , CD13+ eter, CD15+ eter, CD38+, CD71--/+, MPO+/-	CR	early relapse	2 months	4 months

Conclusions. Our population reflects the distribution in cytogenetic risk groups identified in the MRC work. With the use of routine molecular biology we can further stratify our patients, but sometimes the extreme biological complexity in AML may led to slip out from any prognostic score system. Moreover, in our setting, considering the small cohort's size, +8 alterations showed a dismal prognosis and suggest further studies.

PO-067

IDENTIFICATION OF NEW MUTATIONS IN THE CEBPA GENE. THE FLORENCE HEMATOLOGY EXPERIENCE

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Introduction. CCAAT/enhancer binding protein alpha (CEBPA) encodes a transcription factor that regulates neutrophil development and differentiation. Mutations in the CEBPA gene are detected in 5% to 14% of cytogenetically normal acute myeloid leukemia (CN-AML). Typical alterations are N-terminal frameshift mutations and in-frame insertions or deletions in the C-terminal basic leucine zipper (bZIP) region. Some patients show a single mutation CEBPA (CEBPAsm), but most of them have double mutations (CEBPAdm). In the majority of CEBPAdm AML, the mutations are typically on different alleles and involve a combination of a N-terminal and a C-terminal mutation. CEBPA mutations have been associated with a relatively favourable outcome and therefore are emerging as a prognostic marker at the diagnosis. In order to obtain a better insight into the distribution of the various types of CEBPA mutations and evaluate their impact on clinical outcome, we screened for CEBPA a cohort of 208 *de novo* AML patients. *Methods*. We analysed the entire coding region of CEBPA using three overlapping PCR primer pairs designed by Pabst *et al.* (2001) with minor modifications. PCR products were sequenced in both directions on ABI-3100 Genetic Analyzer (Applied Biosystems). *Results*. By sequencing the entire CEBPA coding region, we identified a total of 33 mutations in 23 patients (11%) in our cohort of 208 patients *de novo* CN-AML. Within them, 10 patients had multiple abnormalities (CEBPAdm) while 13 patients had single CEBPA mutation. Among these 33 mutations detected, 16 were already identified in previous studies, while the others (listed in Table 1) had not been described before. The results can be summarized as follows: 8 of the 17 alterations described were frameshift mutations, 8 were insertions/duplications in-frame, 1 was a missense point mutation.

Table 1.

Patient number	FAB	Category	Nucleotide change	Amino acid change	Comment
1.	M2	single	146delC	P49fsX159	Frameshift, new N-terminal stop
2.	M2	Double	65_103del (-43bp)	P22fsX168	Frameshift, new N-terminal stop
2.	M2		919_954dup (+36bp)	N307_T318dup	In-frame insertion in LZ
3.	M2	double	916_945dup (+30bp)	R306_L315dup	In-frame insertion in LZ
4.	M2	double	914_915ins 15bp	T310_Q311insRD HTS	In-frame insertion in LZ
6.	M2	double	291delC	P97fsX159	Frameshift, new N-terminal stop
6.	M2		925_926insAGT CTAAGA	E309_T310insKSK	In-frame insertion in LZ
7.	M1	double	178_188del (-11bp)	T60fsX109	Frameshift, new N-terminal stop
7.	M1		901_924dup (+24bp)	D301_V308dup	In-frame insertion in LZ
8.	M2	double	61_63dupAG	S21fsX159	Frameshift, new N-terminal stop
8.	M2		930_931insAAG	T310_Q311insK	In-frame insertion in LZ
9.	M4	double	97_112del (-16bp)	33_38delFPRGAGf sX159	Frameshift, new N-terminal stop
9.	M4		992subT>C	L331P	Missense substitution in LZ
10.	M2	double	180_186delGTC CATC	T60fsX159	Frameshift, new N-terminal stop
10.	M2		915_948dup (+39bp)	Q305_E316dupQ RNVETQQKVLE	In-frame insertion in LZ
11.	M4	single	61_63dupAG	S21fsX159	Frameshift, new N-terminal stop
14.	M2	single	933_934insCGG	Q311_Q312insR	In-frame insertion in LZ

The nucleotide numbering is according to the GeneBank access number No. NM_004364.2.

According to literature data, insertions/deletions mutations (3n+1 bp) out-of-frame were concentrated in N-terminal region causing frameshift in the reading sequence and involving the formation of a new stop codon. All new mutations identified in the C-terminal region were in-frame and occurred between AA 301 and AA 318. We observed in four cases a "*de novo*" nucleotides insertion, while all the other patients showed long existing sequences duplication to 36 bp. Patient # 14 showed a nonsense nucleotide substitution in position 992 of the C-terminus, in which a thymine was replaced by a cytosine, corresponding to a Leucine-Proline amino acid substitution (L331P), creating a novel stop codon. *Conclusions*. In this analysis on a cohort of 208 *de novo* AML, we identified 17 new CEBPA mutations not described before. Due to the relevant role of CEBPA in leukemogenesis, the identification of novel mutations aids to get more insight into the biology and prognosis of CEBPA-mutated AML subset. A longer follow up is necessary to evaluate the impact of CEPBAdm and particularly of these new mutations on the clinical outcome.

PO-068

'MEMORIAL' PLUS AMIFOSTINE EFFICACY AND FEASIBILITY IN AN ELDERLY COHORT OF 149 AML PATIENTS

Capelli D, Chiarucci M, Saraceni F, Poloni A, Mancini G, Trappolini S, Offidani M, Rupoli S, Montanari M, Scortechini AR, Gini G, Scortechini I, Discepoli G, Leoni P, Olivieri A

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In elderly AML the prognosis still remains poor with only 10% long term OS. Aging is often synonymous of frailty, traducing in difficulties of enrolment of older patients in experimental study. Furthermore the superior incidence of secondary disease and unfavourable cytogenetic is often associated with detrimental outcome. We explored feasibility and efficacy of an high dose Cytarabine regimen, the so called ‚ÄòMemorial', including Idarubicin plus Amifostine, in a cohort of 149 AML patients older than 59 years and prospectively evaluated by Multidimensional Geriatric Assessment, between 1999 and 2010. 91 patients (61%) resulted fully or partially fit with adequate cardiac function and eligible to receive a Memorial based induction and consolidation. The remaining 58 patients received BSC. After Memorial we observed 5 induction deaths (5.5%) and 76% CR rate (67/88, 3 too early) with 67 grade III-IV febrile episodes, 13% and 9% grade III-IV mucositis and liver toxicity. Neutrophils (>1,500/mcl) and platelets (>20,000/mcl) recovery was achieved on days +15 and +16 respectively, with a median duration of hospitalization of 30 days (range: 15-69). Sixty-one patients achieving CR received a consolidation, followed by Allogeneic Transplant in 4, Autologous Transplant in 22 and Gemtuzumab Ozogamycin in 23, according to the mobilization results and donor availability. The remaining patients went off the study because of early leukemia relapse or poor tolerance. With a median follow-up of 70 months we observed a 20% 8-yr OS with a median duration of 11.4 months. The 58 BSC patients had a significantly lower OS since all died within 18 months with a median OS of 1.5 months (p<0.001). Poor risk cytogenetic was confirmed as the only factor predictive of refractory disease with a RR of 3.26 (p=0.03). By the way we observed a 63.6% CR rate, unusually high if compared to the results reported by the literature. 8-yr OS was significantly reduced at univariate analysis in patients with secondary disease *vs* those with *de novo* AML (10.4% *vs* 27.1%; p=0.04); in those with unfavourable cytogenetic risk *vs* other risk groups (11.4% *vs* 30.3% p=0.01) and in those with WBC count> 50,000/mcl *vs* < 50,000/mcl (0% at 33 months *vs* 23.4%, p=0.009) (Figure 1).

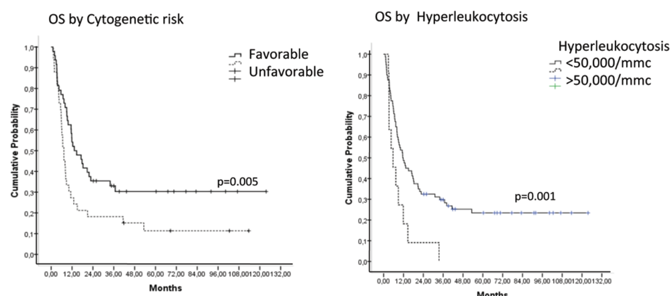

Figure 1. OS by Cytogenetic and Hyperleukocytosis.

Patients with poor karyotype and hyperleukocytosis ≥50,000/mcl had respectively 2.1 (p=0.005) and 3.53 (p=0.001) RR to die when compared with other patients at multivariate analysis. In conclusion Multidimensional Geriatric assessment allows a reproducible and accurated selection of AML elderly patients eligible for intensive chemotherapy treat-

ment. Memorial plus Amifostine represents an intriguing approach in elderly AML patients with an acceptable haematological and extra-haematological tolerance, a transplant feasibility comparable to other studies; finally 8-yr OS doubled that reported by the literature even in unfavourable setting with secondary disease and poor risk cytogenetic.

PO-069

ARSENIC TRIOXIDE (ATO) DOWNREGULATES LEUKEMOGENESIS-ASSOCIATED GENES IN AML WITH NPM1 GENE MUTATION

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Introduction. Acute myeloid leukemia (AML) is a clonal disorder of the hematopoietic precursor cell. Nucleophosmin (NPM1) gene mutation is the most common genetic abnormality in adult AML accounting for about 30% of cases. We have previously shown that NPM1 mutant protein was downregulated by arsenic trioxide (ATO), at least partially by post-transcriptional mechanisms. NPM1 mutant expression is associated with a peculiar gene expression profiling in AML. Hox genes encode for homeodomain transcription factors that are highly conserved, and are known to be important in both normal hemopoiesis and leukemogenesis and overexpressed or rearranged in a variety of leukemias. Among others, HOXA9, HOXA10 and MEIS1 genes are upregulated in AML with NPM1 mutation. FLT3 (CD135) is a transmembrane protein involved in cell survival and proliferation that is also frequently overexpressed or constitutively activated (FLT3-internal tandem duplication) in AML, including NPM1-mutated AML. *Materials and Methods.* OCI-AML3 human leukemia cells (bearing NPM1 mutation, but no FLT3-ITD mutation) were used as *in vitro* model to study the effects of pharmacological doses (3 microM) of arsenic trioxide (ATO). Upon treatment, RNA from OCI-AML3 was isolated and retrotranscribed and the resulting cDNA used for quantitative or semiquantitative PCR for detection of mutant NPM1, HOXA9, HOXA10, MEIS 1 and FLT3. In addition to RT-PCR, NPM1 mutant and FLT3 (CD135) expression was also evaluated at protein level, respectively by western blot with specific an anti-NPM1 mutant antibody and by flow-cytometry using a CD135-PE antibody and CD135 median fluorescence intensity (MedFI) calculated and reported. *Results*. After 24 hours, ATO induced a significant downregulation of NPM1 mutant protein and gene. Interestingly, this was associated with significant downregulation of HOXA9 (NT 0,86 *vs* ATO3 0,56-evaluated as ratio gene/GAPDH), HOXA10 (NT 0,66 *vs* ATO3 0,32 evaluated as ratio gene/GAPDH) , Meis1 (NT 0,83 *vs* ATO3 0,55- evaluated as ratio gene/GAPDH) and FLT3 (NT 0,73 *vs* ATO3 0,3- evaluated as ratio gene/GAPDH) genes. Moreover, at the same timepoint , reduction in FLT3 (CD135) protein cell surface expression levels was demonstrated by flow cytometry (MedFI 33444 -ÔÉ† 530 arbitrary units). *Conclusions*. Pharmacological doses of ATO downregulate mutant NPM1 protein and gene leading to downregulation of HOXA9, HOXA10, Meis1 and FLT3 genes expression in OCI-AML3 NPM1 mutated cell line. We have already reported some degree of cellular differentiation in OCI-AML3 cells treated with ATO and it is still unclear if downregulation of Hox genes is secondary to ATO or to differentiation itself. However ATO treatment seems to target several important genes involved in leukemogenesis, cell survival and proliferation, making this compound an attractive option for further *in vitro* and *in vivo* experiments in NPM1 mutated AMLs.

PO-070

ARSENIC TRIOXIDE AND ALL-TRANS-RETINOIC ACID MEDIATE DOWNREGULATION OF MUTATED NPM1 AND SENSITIZE NPM1-MUTATED AML CELLS TO DAUNORUBICIN

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Introduction. Standard chemotherapy±hemopoietic-stem-cell transplantation can cure acute myeloid leukemia (AML) in 40-50% of younger adults and in 10-15% of elderly patients (>60 years old). Therefore, novel therapeutic approaches are needed to improve patient outcomes. NPM1-mutated AML accounts for about 30% of adult AML. Because NPM1 mutations are common and represent a founder genetic lesion in AML, they are an appealing therapeutic target. Efforts are ongoing to identify drugs which could interfere with the mutant and residual wild-type NPM1 levels and/or functions. We previously reported that ATRA induced down-regulation of NPM1 mutant protein in NPM1 mutated cells and we recently showed that ATO induces degradation of NPM1 leukemic protein and apoptosis in leukemic cells. In this study, we explored whether these compounds could also exert a combined action on the NPM1 protein, more specifically in the setting of NPM1-mutated AML, and if this combination would sensitize cells to standard Daunorubicin chemotherapy. *Methods.* We tested pharmacological doses of ATO (1-3 μM), ATRA (1 μM) and sub- pharmacological doses of DNR (0,05 μM) in the human AML cell lines OCI/AML3 (bearing mutated NPM1) for induction of apoptosis, NPM1 protein downregulation and chemosensitization. *Results.* We confirmed that, in NPM1-mutated OCI/AML3 cells, ATO and ATRA induced and marked down-regulation of NPM1 mutant (NPMm) protein, leading to apoptosis, whilst the NPM1 wild-type (NPMwt) protein levels were less affected. Strikingly, combination of ATO and ATRA synergistically induced a significant level of apoptosis in AML3 cells (*t-test, p=0,014) and a significant decrease in NPMm protein level as compared to the single treatments (Figure 1A). Remarkably, pretreatment with a combination of ATO and ATRA induced a significant increase in Daunorubicin-induced apoptosis (* t-test, p=0,011) as compared to ATO and/or ATRA, and to Daunorubicin single treatments, indicating that ATO and ATRA sensitize AML3 cells to this antracyclinic drug (Figure 1B). *Conclusions.* We conclude that ATO and ATRA combined treatment induce a significant down-regulation of mutated NPM1 protein as compared to the single treatments resulting in a significantly higher cell apoptosis. Moreover, ATO and ATRA combined pre-treatment sensitize AML3 cells to suboptimal doses of Daunorubicin, a chemotherapeutic drug commonly used in AML treatment. These findings provide a rationale for further exploiting these compounds in combination in clinics for the treatment of NPM1-mutated AML.

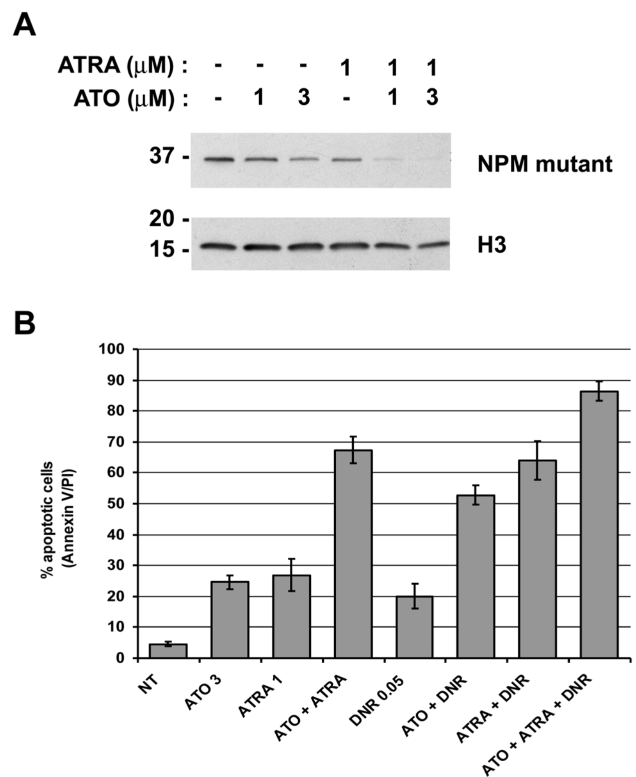

Figure 1.

PO-071

DESFERRIOXAMINE INDUCES APOPTOTIC AND DIFFERENTIATING EFFECTS ON OCI/AML3 CELL LINES HARBORING NPM1 MUTATIONS

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Background. Downregulation of NPM1 protein in NPM1-mutated acute myeloid leukemia (AML) represents an attractive and promising

approach in order to tailor the treatment of patients affected by this peculiar AML subtype. Previous investigations have shown that Desferrioxamine (DFO) is able to modulate NPM1 location in leukemic cell lines and is also able to induce differentiation in myeloid cells interfering with the reduced oxygen species (ROS) levels. Since NPM1 mutation represents the main genetic lesion in OCI/AML3 cell line, we aimed at studying the biological effect of DFO in this peculiar cell line. *Methods*. Human OCI/AML3 cell line were treated with continous exposition of scaling doses of DFO. EC50 was evaluated with WST assay. Wild-type (wt) and mutated (mut) NPM proteins together with cell cycle checkpoint proteins expressions were evaluated by western blot. Apoptosis was studied using Annexin-V/Propidium Iodide cytometric assay (AnxAV/PI). Additionally, differentiation was studied with the following antibodies combination: CD14-FITC/CD11b-PE/CD66b-PerC/CD33-APC. Samples were collected after 4, 24, 48 and 72 hours incubation. Statistical analysis was performed with two-way ANOVA test; significance levels was set as p=0.05. *Results*. WST assay showed a DFO-EC50 of 180 mcMol. Accordingly, apoptosis assay of cells treated with DFO 150 and 200 mcMol revealed a viability halved compared to control in both conditions after 24h DFO exposure. However, taking into account *in vivo* pharmacokinetics data in the literature, we evaluated the activity of 10 and 20 mcMol DFO that, at WST assay, seemed to determine at least a reduced metabolic activity compared to control. After 72h exposure, AnxAV/PI revealed a time-depended reduced viability (85% and 39% for 10 and 20 mcMol respectively compared to 96% viability of control, p=0,03). Additionally, a threefold augmented CD66b expression was noted after 72h only in cells treated with DFO 20 mcMol. CD11b and CD14 expression was significantly augmented after 48 and 72h exposures in both conditions compared to control (p<0,01). Interestingly, after 48 and 72 h, WB analysis showed a reduction in NPMmut and NPMwt levels together with increased expression of DNA damage markers (*i.e.* pATM, Chk1 and pH2Ax). *Conclusions*. *In vitro* tests indicate potential effects of DFO on leukemic cells harbouring NPM1 mutation. Indeed, DFO (at clinically safe concentrations) was able to reduce the viability of leukemic cells determining time-dependent differentiation associated with downregulation of NPMmut and NPMwt expression.

PO-072

ASSOCIATION BETWEEN TP53 rs1042522 (P72R) POLYMORPHISM AND ADULT B CELL ACUTE LYMPHOBLASTIC LEUKEMIA PATIENT (B-ALL).

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Introduction. The reported TP53 mutation rate in hematological system cancers is relatively low compared to other cancer types (11.03%) and in ALL is even lower (5-8%). Many mutations and single nucleotide polymorphisms (SNPs) in TP53 have been associated with cancer. Of the 100 known TP53 SNPs, only few have been well characterized in terms of distribution, functions and association with cancer risk. The most widely studied is the rs1042522 at codon 72, which is a proline (P)/arginine (R) variant. The P72 and the R72 polymorphic forms differ in protein structure and consequently in molecular and biological activities. Aims: Since the pattern, frequency and significance of rs1042522 in adult B-ALL subjects of European ancestry needs still to be determined, we analyzed 176 adult B-ALL patient samples to address this issue. Furthermore, in 53 cases, we compared the leukemic patient sample to its paired germline material, to better understand the hereditary role of this SNP. Materials and *Methods.* TP53 exon 4 screening, where the rs1042522 SNP is located, was performed on DNA from 263 samples (136 diagnosis, 74 relapse and 53 germline/saliva). Comparison of rs1042522 genotype and allele frequencies between B-ALL and 379 healthy European 1,000 Genomes Project subjects was performed. *Results.* By PCR and subsequent Sanger sequencing, the P72R SNP was investigated in 176 adult B-ALL samples, in particular 136 pts were analyzed at the diagnosis and 40 at the relapse. The diagnostic samples were found heterozygous in 34.6% (47/136, C/G), homozygous for arginine in 61% (83/176, G/G) and homozygous for proline in 4.4% (6/176; C/G) of cases. The relapse samples presented 1/40 (2.5%) C/C, 19/40 (47.5%) C/G and 20/40 (50%) G/G genotype. Considering all samples, we observed P72 in 4% (7/176) , P72R in 37.5% (66/176) and R72 in 58.5% (103/176). A significant increased frequency of rs1042522 G allele was observed in disease subjects (77.3%) with respect to healthy ones (28.5%) (Fisher's Exact test p=9.74 x 10-54, OR=8.53), in line with an over-representation of GG homozygotes in B-ALL. This suggests that the G allele could represent a B-ALL risk factor in populations of European ancestry or that this association signal reflects its high LD with actual causative alleles (*e.g.* TP53 rs1642785). In the 53 germinal samples the 3 possible polymorphism genotypes were represented as follow: C/C in 3/53 (5.7%), C/G in 19/53 (35.8%) and G/G in 31/53 (58.5%) samples. *Conclusions.* This analysis suggests that P72R SNP may be strongly associated with the risk of B-ALL in European adults. However further studies based on larger sample size are needed to confirm these conclusions. Supported by: EuropeanLeukemiaNet, AIL, AIRC, PRIN 2010-2011, Fondazione del Monte di Bologna e Ravenna,European Union Seventh Framework Programme [FP7/2007-2013].

PO-073

HOW TO GAIN CYTOSINE ARABINOSIDE MILLIGRAMS

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Introduction. Cytarabine (Ara-C) a deoxycytidine analogue, is one of the most active drugs in the treatment of acute leukemias and is widely applied at a variety of other hematological malignancies. Although chemical and physical in-use stability of commercial Ara-C has been recommended for 48 hours at room temperature after dilution, from a microbiological point of view (data from drug informative sheet of Aracytin, available in Italy), the product should be used immediately. The cancer drugs preparation and dispensing in designated centralized chemotherapy (CHT) preparation pharmacy units (CPU) has been extensively studied to improve its quality and minimize personnel exposure to these drugs. We know relative little about the possibility to optimize and economize the use of CHT agents in CPU. *Aims*. Here we explore the chemical and microbiological stability of a commercial Ara-C sample (Aracytin) in its pharmaceutical form after its reconstitution and storage at 4°C, in order to save drug. *Methods.* Commercial Ara-C has been investigated under the common conditions of the clinical use to be degradable in water as in physiological solution. Ara-C samples reconstituted according to the commercial reported instructions and stored at 4°C, in controlled and validated aseptic conditions, were analyzed by high-field nuclear magnetic resonance spectroscopy (NMR). The results obtained were compared with those derived from identical samples bubbled with air at 4 °C, and 25 °C, at different time and concentrations, respectively. Microbiological assay, applying the cylinder-plate method, was performed. After an initial entry through the vial closure with a sterile syringe with needle, aliquots of reconstituted and unused solution were withdrawn after multiple aseptic entries trough the cap at 1 to 5 days and were assayed against solutions of reference standard. *Results.* All the samples remained chemical unchanged for one month. Low temperature and scarce contact with air decrease the degradation process. We demonstrate the absence of any microbiological contamination, respecting the described aseptic condition, for at least 5 days. *Conclusions.* We demonstrate chemical and microbiological stability of Ara-C solutions for at least 30 and 5 days respectively, allowing the use of the same vial for a prolonged time. It seems to be cheap and particularly useful for CPU and hospital, in order to economize without losing in efficacy and safety. We can also auspicate the use of the same vial, after a specific training about aseptic and sterile procedures to collect multiple doses, for outpatients who receive subcutaneously daily administration of low doses of Ara-C for more than a week. It could avoid to discard a great amount of drug and allow the outpatient to self administrate drug without daily return to the Hospital only for a simple and short-term injection. The opportunity to save Ara-C milligrams could positively impact both on health economic and patients quality of life.

Chronic Myeloid Leukemia and Myeloproliferative Disorders

PO-074

CARDIOVASCULAR EVENTS: RISK FACTORS AND CORRELATIONS WITH THE JAK2V617F ALLELE BURDEN IN PATIENTS WITH Ph'-NEGATIVE MYELOPROLIFERATIVE NEOPLASMS. A MONOCENTRIC STUDY

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Introduction. Myeloproliferative neoplasms (MPN) are a group of clonal diseases associated with JAK2 mutation (V617F) in a percentage varying from 50% (Essential Thrombocytemia and Myelofibrosis) to 95% (Polycitemia Vera). Treatment targets are represented by the maintenance of an adequate blood count, the reduction of the systemic symptoms, and the prevention of the cardiovascular events. Recent papers reported an association between the JAK2V617F mutation and the venous/arterial thromboses, although many studies are still in progress in order to assess whether this parameter can be considered to play an independent role in patients that often present comorbidities or already known cardiovascular risk factors. *Aims*. To correlate the presence of the JAK2 mutation, or its mutated allele burden, to the vascular events in MPN patients and to weight the JAK2 significance in respect of the cardio-vascular risk factors. *Methods.* We analyzed 384 patients (median age 68 years; range 20-89) affected by Ph'-negative neoplasms for the JAK2V617F mutation by using the JAK2 MutaScreen kit, that is based on the TaqMan allelic discrimination technique. In the mutated cases, the JAK2 MutaQuant kit was used to quantitate the mutated allele (Ipsogen, Luminy Biotech, Marseille, France). The sensitivity of these tests was 1x10-4. Some of our cases have been also assessed by mean of a new technique, the droplet digital PCR (dPCR, BIO-RAD), that allows the quantitation of the mutated allele without any reference curve. *Results*. The JAK2V617F mutation has been reported in 222 (58%) of our patients: in the 57% of the patients with Essential Thrombocythemia (ET), 77% with Polycitemia Vera (PV) and 62% with Myelofibrosis (MF). The mean allele burden was 39.5%: the percentage of the mutated allele was significantly highest in the MF, followed by the PV and then by the ET ($p<0.01$), and it significantly correlated with the WBC count ($p=0.02$). Cardiovascular events have been reported in 137 out of the 222 mutated patients (62%); they were mainly distributed on the arterial side (52%), in particular myocardial infarction (35%), ictus cerebri (24%), and ischemic transitory attack (14%). Among venous events, the deep venous thromboses (44%) were predominant. The events were significantly correlated with the high risk of diseases at diagnosis ($p<0.01$), a PADUA score >4 ($p<0.01$), and dyslipidemia ($p=0.04$). On the contrary, their occurrence was independent from the smoke, diabetes, and hypertension. In addition, vascular events did not correlate with leucocyte count, hematocrit, platelet, and JAK2 allele burden (Table 1). *Conclusions*. Our study showed that more than the half of patients affected by MPN presented cardio-vascular events. Their occurrence was higher in cases with WBC>11000/uL, high risk diseases, and in those showing a high PADUA score. The mutated allele burden, measured by both real-time and droplet PCR, did not significantly condition the occurrence of cardio-vascular events.

Table 1. Characteristics of the patients,

N° patients	384
Age	20-89 (median 65)
Gender	
M	177 (46%)
F	207 (54%)
Diagnosis	
PV	82 (21%)
TE	218 (57%)
MF	70 (18%)
Not Determined	14 (4%)
JAK2V617F	222/384 (58%)
JAK2V617F with vascular events	137/222 (62%)

PO-075

mRNA-miRNA INTEGRATIVE ANALYSIS IN PRIMARY MYELOFIBROSIS CD34+ CELLS UNCOVERS THE ROLE OF miR-155/JARID2 AXIS IN ABNORMAL MEGAKARYOPOIESIS

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Introduction. Primary myelofibrosis (PMF) is a myeloproliferative neoplasm (MPN) characterized by bone marrow fibrosis, extramedullary hematopoiesis and megakaryocyte hyperplasia. Despite the discovery of somatic mutations in JAK2, MPL and CALR genes, the molecular framework underlying PMF pathogenesis remains to be fully elucidated. Moreover, abnormal microRNA (miRNA) expression seems to add to the molecular complexity of MPNs, as specific miRNA signatures that discriminate MPN cells from those of normal donors were previously reported. *Methods.* In this study, we analyzed both gene and miRNA expression profiles in CD34+ cells from 31 healthy donors and 42 PMF patients using Affymetrix technology (HG-U219 and miRNA 2.0 arrays). Then, in order to construct regulatory networks involving functional miRNA-target interactions, we performed an integrated analysis with Ingenuity Pathway Analysis (IPA) software, which combines the miRNA expression profile with computational predicted targets and gene expression data. *Results.* Microarray analysis revealed higher expression levels of several putative cancer markers (WT1, ANGPT1) and oncomiRs (miR-155-5p, miRNAs of the miR-17-92 cluster) in PMF samples when compared with the normal counterparts. Furthermore, several genes related to PMF progression, as involved in megakaryocyte (MK) differentiation (NFE2, CD9, miR-34a-5p), fibrosis development (LEPR, MMP9) and aberrant migration (RHOB, ARHGAP18), were significantly upregulated in PMF cells. Then, by miRNA-gene integrative approach, we identified several networks involved in dysregulation of transcriptional control and chromatin remodeling, potentially related to PMF pathogenesis. In particular, we identified an interaction network that includes several upregulated oncomiRs (miR-155-5p, miR-29a-3p) and their targets, like NR4A3, CDC42, HMGB3, already described as involved in myeloproliferative phenotypes, and the chromatin remodeler JARID2, which is frequently deleted in leukemic transformation of chronic myeloid malignancies. Since validation of 3'UTR-miRNA interactions by luciferase reporter assays showed JARID2/miR-155-5p as the strongest relationship of the network, we investigated the function of this pair in normal CD34+ cells. Of note, miR-155-5p overexpression in CD34+ cells, as well as siRNA-mediated JARID2 inhibition, causes a significant increase of CD41+ cells compared with control and a remarkable increase in CFU-MK colonies. Moreover, morphological analysis showed that miR-155-5p overexpression and JARID2 silencing induce a considerable enrichment in MK precursors at different stages of maturation. *Conclusions.* This study allowed the identification of different networks possibly involved in PMF onset and progression. In particular, we demonstrated that miR-155-5p upregulation reproduces a hyperplastic megakaryopoiesis in CD34+ cells through the downregulation of JARID2, suggesting the involvement of miR-155/JARID2 axis in the PMF pathogenesis.

PO-076

DNA METHYLTRANSFERASE 1 DRIVES TRANSCRIPTIONAL DOWN-MODULATION OF BETA CATENIN ANTAGONIST CHIBBY1 ASSOCIATED WITH THE BCR-ABL1 GENE OF CHRONIC MYELOID LEUKEMIA

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Introduction. Chibby1 is an antagonist of β catenin, a central component of chronic myeloid leukemia pathogenesis and progression to blast crisis. Our recently published study proved that Chibby1 down-modulation is a component of β catenin activation associated with the BCR-ABL1 fusion gene, partly contingent upon transcriptional events and driven by DNA hyper-methylation at promoter-associated CpG islands of the Chibby1-encoding gene C22orf2. Moreover, it established that Chibby1 induction in response to imatinib in chronic myeloid leukemia cells proceeds, at least partly, from C22orf2 promoter de-methylation. Indeed, methylator phenotype encompassing genes involved in leukemic cell proliferation and survival is a common event in chronic myeloid leukemia, eventually associated with the disease progression and drug resistance outcome. *Methods*. PCR amplification of DNA from chromatin immunoprecipitation products was used to assess the amounts of DNA methyltransferase 1 and 5 methylcytosine at a 205 bp promoter region encompassing nucleotides -85 to +120 of C22orf2 promoter and at a 342 bp sequence of the pro-apoptotic BCL2- like11 (BIM) in BCR-ABL1+ cell line K562 and mononuclear cell fractions from bone marrow samples of imatinib-responsive and -resistant chronic myeloid leukemia patients. *Results*. We found that DNA methyltransferase 1 enhanced recruitment is involved in hyper-methylation of C22orf2 promoter, associated with Chibby1 transcript reduction in three out of five imatinib-responsive patients and all three imatinib-resistant patients, supporting that such epigenetic modification is not the cause of overt resistance to imatinib. Further investigation showing that the DNA methyltransferase 1-driven hyper-methylation of the pro-apoptotic BCL2-like11, a crucial gene for the prognosis and response of chronic myeloid leukemia to imatinib , is not strictly associated with drug resistance. *Conclusions*. Those findings suggest that DNA methyltransferase 1-driven epigenetic control on Chibby1 and other tumor suppressor genes such as BCL2-like11 is not the cause of overt chronic myeloid leukemia resistance to imatinib. Still, the DNMT1-driven hyper-methylation at those critical genes for cell survival and proliferation may contribute to the disease clonal evolution, hence supporting the clinical use of de-methylating agents, in particular, in the disease advanced stages.

PO-077

INTEGRATED ANALYSIS OF GENE EXPRESSION AND COPY NUMBER DATA: A NOVEL APPROACH TO UNRAVEL NEW PATHOGENIC MECHANISMS IN PRIMARY MYELOFIBROSIS

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Introduction. Myeloproliferative neoplasms (MPNs), that include polycytemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF), are clonal hematopoietic stem cells disorders characterized by increased proliferation of terminally differentiated myeloid cells. Beginning in early 2005, a number of novel mutations involving JAK2, CALR, MPL, TET2, ASXL1, CBL, IDH and IKZF1 have been described in MPNs. However, it is not yet clear whether and how these abnormalities contribute to disease initiation, clonal evolution or leukemic transformation. *Methods.* To get new insights into the primary genetic lesions involved in PMF pathogenesis, we performed an integrative analysis of genotype and copy number data with global gene expression data. Gene expression profiling (GEP) was performed by means of Affymetrix HG-U133 plus 2.0 arrays on the same patient samples already characterized for genotyping and copy number analysis on the Cytoscan HD arrays. Transcriptional and copy number profiles were analyzed using SODEGIR (Significant Overlaps of Differentially Expressed and Genomic Imbalanced Regions), a bioinformatics procedure that allows the integration of copy number, obtained from SNP mapping arrays, with transcriptional data. *Results.* The integration of genomic and transcriptomic data from the same samples allowed identifying specific genomic regions with concordant alterations in DNA and mRNA profiles, thus providing a powerful tool to identify genes relevant in PMF pathogenesis. In particular, in the set of amplified genes we found PXDN, a protein involved in extracellular matrix remodeling, which could play a role in the development of the bone marrow fibrosis characterizing PMF patients. On the other hand, in the signature of deleted genes, we found several zinc finger proteins such as ZNF92, ZNF107, and ZNF430, whose function is worth of further characterization in the contest of PMF pathogenesis, and HMGXB4 and ZNF431, *i.e.*, some negative regulators of WNT and Hh pathways, respectively. Finally, among deleted genes we also identified YTHDC1, a splicing factor whose loss has been recently associated with poor overall and disease-specific survival in endometrial cancer. Since mutations of the spliceosome machinery have been recently described in myelodysplastic syndromes, the loss of YTHDC1 locus in PMF patients is likely to play a role in MPN pathogenesis. *Conclusions.* Our preliminary results suggest that this novel approach of integrated genotype and gene expression analysis could represent a powerful tool to unravel novel pathogenetic mechanisms underlying PMF development.

PO-078

EXTREME THROMBOCYTOSIS IN CHRONIC MYELOID LEUKEMIA (CML) IN THE ERA OF TYROSINE KINASE INHIBITORS (TKIS)

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Introduction. Thrombocytosis is a common feature in chronic myeloproliferative disorders. The incidence of thrombocytosis in CML is reported to be around 30 to 50%. Extreme thrombocytosis defined as a platelet (PLT) count > 1.000x10^9/l is uncommon in CML as well as isolated thrombocytosis. *Methods.* From January 2001 to April 2014 we treated 308 consecutive patients (pts) with CML focusing on the clinical management and the therapeutic response of CML with extreme thrombocytosis. *Results.* Thirty pts (9.7%) presented at diagnosis an extreme thrombocytosis. There were 18 females and 12 males with a median age of 58 years. At diagnosis, median hemoglobin level was 11.6 g/dl, median WBC 31.4 x 10^9/l and PLT count 1.576 x 10^9/l. The Sokal score was high in 17, intermediate in 8 and low in 5 pts. In all cases PCR analysis showed the presence of p210 and the absence of JAK2 V617F mutation. All but three pts received initial treatment with hydroxyurea and allopurinol. One patient underwent PLT apheresis. PLT count was generally unresponsive to initial treatment. Upfront treatment was imatinib in 25 pts and nilotinib in 5 pts and PLT count normalization was rapidly achieved after introduction of TKIs. Haematological response was reached at a median of 1 month, complete cytogenetic response after 3 months and major molecular response in 23 out of 25 pts after 9 months (range 3-44). One patient was in suboptimal molecular response at 18 months of imatinib and he was shifted to dasatinib, achieving MMR. One patient, without bcr-abl mutation, lost MMR after 5 years of imatinib, and shifted to nilotinib achieving MMR. Three pts showed resistance to imatinib after 1, 3 and 7 years respectively and shifted to nilotinib. One pt is now in therapy with ponatinib after showing resistance to nilotinib. All pts, except one dead for solid cancer, are alive at a median follow up of 70 months. *Conclusions.* Extreme thrombocytosis in CML is rare. Prolonged bleeding time was detected in all pts although it was not accompanied by bleeding diathesis. Cytoreduction with hydroxiurea was not able to achieve normalization of PLT count, however pts with extreme thrombocytosis were easily and rapidly managed by TKIs.

PO-079

THE COMBINATION OF PANOBINOSTAT AND RUXOLITINIB EXERTS SYNERGISTIC EFFECTS TO OVERCOME BONE MARROW (BM) STROMA PROTECTION IN HUMAN MYELOPROLIFERATIVE NEOPLASTIC PRIMARY PROGENITOR CELLS

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Introduction. The JAK inhibitor ruxolitinib decrease spleen size and alleviate constitutional symptoms in myelofibrosis (MF) patients. Thus, this drug represents a milestone in the treatment of MF patients, even though non-curative. The pan-histone deacetylase inhibitor panobinostat, in a phase I study for patients with MF, showed to be clinically active, although lower than expected. In this scenario, BM stromal cell compo-

nents create a favourable pathologic microenvironment in MF that nurtures and protects the malignant cells. In this regard, we prove that the cytotoxic activity of both ruxolitinib and panobinostat is significantly inhibited by BM stromal soluble factors, and that the combination of the two inhibitors synergizes to overcome the observed BM stroma related resistance. *Methods*. JAK2V617F tumor cell lines HEL and SET2 were treated with ruxolitinib and panobinostat in RPMI medium, defined as regular media (RM), or on monolayers of stroma cell line HS-5 or BM stroma secreted cytokines, defined as HS5/Stroma Conditioned Media (HS5/SCM). Isolated BM progenitors MPNs cells were treated with both drugs in RM or HS5/SCM. *Results*. In RM panobinostat or ruxolitinib induce a significant apoptosis in SET2 and HEL cells in a dose-dependent manner. Indeed, The IC50 of SET2 cells treated with panobinostat or ruxolitinib is significantly increased in the presence of HS-5/SCM (31nM and 1222nM, respectively) *versus* the IC50 in RM (11nM and 305nM, respectively). In contrast, HEL cell line shows a great resistance to ruxolitinib treatment, since only a high concentration of drug (1000nM) is able to induce a significant apoptosis. The IC50 of HEL cells treated with panobinostat increased from 32nM to 66nM. The combined drugs panobinostat and ruxolitinib strongly synergizes, increasing SET2 (96%±1%) and HEL (73%±5%) apoptosis, regardless HS5/SCM exposition. Treatment of SET2 cells with ruxolitinib reduced phosphorylation of the JAK downstream target STAT3, whereas panobinostat reduced both phosphorylation and total STAT3/STAT5 levels. Moreover, the combination treatment completely abrogates STAT5 expression and induced a further inhibition of pSTAT3 in SET2 cells. Moreover, the co-treatment of panobinostat and ruxolitinib is also capable to reduce viability of CD34+ cells derived from seven PMF patients lacking of clinical improvements after at least six months of ruxolitinib treatment. Finally although co-treatment of MPN-CD34+ cells with panobinostat and ruxolitinib is not synergic to reducing CFU outgrowth respect the single agent panobinostat, is significantly effective to reduce the count of MPNs CD34+ cells in long term sub-cultures. *Conclusions*. Our data strongly support the hypothesis that a novel drug combination of ruxolitinib and panobinostat may be more effective in eradicating MPNs cells crammed in the BM rather than the single agent, and may significantly improve response for patients with refractory disease and decrease the rate of therapy resistance.

PO-080

BCR/ABL LEVELS AT ONSET AND RATE OF REDUCTION AT 3 MONTHS OF TREATMENT ARE USEFUL TOOLS TO STRATIFY PATIENTS WITH CHRONIC MYELOID LEUKEMIA (CML)

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Introduction. According to the European Leukemia Net criteria, CML patients have optimal response and better outcome if bcr/abl is <10% at 3 months and <1% at 6 months of therapy. We investigated the role of tumor burden at diagnosis and bcr/abl reduction rate at three months of treatment in predicting response to therapy. *Methods.* Blood and bone marrow samples collected from 16 CML patients have been analyzed. Median age was 62.5(range 30-80); all patients received Imatinib as first line therapy. The median follow up was 33 (7-63) months. RQ-PCR was performed at diagnosis and during treatment. Patients losing the major molecular response and requiring a therapy switch were defined as a failure. RESULTS: 8 out of 16 patients treated with Imatinib changed drug during follow up (group SHIFT-S). Median time between onset and drug change was 20.5 (3-48) months, while median duration of follow up in 8 patients who didn't change therapy (group NO SHIFT-NS) was 27(9-45). In group NS, average of bcr/abl at onset resulted 62.93% *vs* 120.325% of group S; distributions of the 2 samples are significantly different and the median value of bcr/abl at diagnosis was 65.9%(38.4-90.9) in NS group *vs* 82.5%(54.8-290.2) in S group (Fig 1). Moreover, we stratified all 16 patients on the basis of an experimentally defined cut-off of 77% bcr/abl at onset. 6 patients showed value> 77%: all but one patient switched drug during follow up (83%). Only 30% (3/10) patients with a value<77% switched drug. We evaluated bcr/abl reduction rate at 3 months of treatment (molecular values at onset/molecular values at 3 months). The mean ratio was 18.8 (3.08-40.9) in S group *vs* 39(2.9-114) in NS group but median were comparable. *Conclusions*. We hypothesize that in addition to SOKAl risk, bcr/abl values at onset and its reductiona at 3rd month may predict aggressiveness of CML and the need of an early switch from Imatinib to a second generation TKI. Imatinib could be administered to patients with < 77% of bcr/abl transcript at onset; in the other cases a first line with second generation TK inhibitor could be employed to obtain a faster and deeper response.

PO-081

"REAL-LIFE" FRONTLINE DASATINIB TREATMENT IN UNSELECTED ELDERLY PATIENTS WITH CHRONIC MYELOID LEUKEMIA

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Introduction. Dasatinib has been very recently licensed for first line treatment of patients with chronic myeloid leukemia (CML). However, there are still no data available as to toxicity and efficacy of dasatinib in elderly unselected CML patients. *Methods*. To address this issue, we revised a "real-life" cohort of 39 CML patients in chronic phase aged > 65 years treated with frontline dasatinib in 19 Italian Centers from 6/2012 to 3/2014 as concern toxicity and efficacy data. The main clinical features of the patients at diagnosis were as follows: M/F 19/20 (48.7%/51.3%), median age 73.9 years [interquartile range (IQR) 69.8 – 78.4), median Hb 12.3 g/dl (IQR 11.0 – 13.6), median WBC 51.1 x 109/l (IQR 27.8 – 93.9), median PLTS 481 x 109/l (IQR 269 – 746). According to Sokal risk classification, 1 patient (2.5%) was at low risk, 24 patients (61.5%) were at intermediate risk, 10 (25.6%) at high risk and 4 (10.4%) were not classificable. 19/39 patients (48.7%) had ≥ 2 comorbidities requiring concomitant therapies: according to ECOG scale, performance status at baseline was 0 – 1 in 32 patients (82.1%) and 2 in 7 patients (17.9%). *Results*. Median interval from diagnosis to dasatinib start was 22 days (IQR 15 – 30). Starting dose of dasatinib was 140 mg/day in 1 patient (2.6%), 100 mg/day in 29 patients (74.3%) and < 100 mg/day in 9 patients (23.1%), respectively. After a median period of treatment of 8.0 months (IQR 2.8 – 15.8) all patients were evaluable for toxicity; on the whole, grade 3 – 4 hematological and extra-hematological toxicities were reported in 4 (10.3%) and 5 (12.8%) patients, respectively. Overall, 6 patients (15.3%) permanently discontinued dasatinib due to toxicity (2 patients in the first 3-month period of treatment and 4 beyond that period). Pleural effusions of all WHO grades occurred in 7 patients (17.9%): in 2 of them the pleural effusion occurred during the first 3-month period of treatment. As to treatment efficacy, 7 patients were considered too early (< 3 months of treatment) and 32 were evaluable for cumulative response; on the whole, 27/32 patients (84.3%) achieved complete cytogenetic response and 20/32 (62.5%) also a major molecular response. *Conclusions*. Present data shows that dasatinib could have a major role in the treatment of unselected patients aged > 65 years; in particular, dasatinib seems very effective and has a favourable safety profile also in elderly subjects with severe comorbidities.

PO-082

OVEREXPRESSION OF PLATELET FIBRINOGEN RECEPTOR IN PATIENTS WITH PHILADELPHIA-NEGATIVE CHRONIC MYELOPROLIFERATIVE NEOPLASMS RECEIVING ASPIRIN PROPHYLAXIS

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Introduction. Although patients with Philadelphia-negative chronic myeloproliferative neoplasms (MPNs) are considered at high risk of thrombohaemorrhagic events, the importance of platelet count in the assessment of the risk of vascular events is still controversial. More studies on platelet function are needed to identify potential drugs for more accurate antiplatelet action. Platelet fibrinogen receptors (PFRs) are easily detectable by the PAC-1 antibody in flow cytometry, and their expression is unique in the process of platelet activation. The inhibition of platelet cyclooxygenase does not seem to affect PFR expression, which could represent, according to some authors, an underlying mechanism of aspirin resistance. *Methods*. In the present study, blood samples were obtained from 27 consecutive MPN patients who had never received cytoreductive agents. 18/27 patients were undergoing continuous antiplatelet therapy with low-dose aspirin (75-100 mg) at the time of blood collection. Our aim was to evaluate PFR expression in the two different groups. In each experiment, sodium citrate and heparin (positive control for platelet activation) tubes were used. Within 10 minutes of blood sampling, 5 ml of whole blood from each tube was incubated for 20 minutes at room temperature in the dark at saturating concentrations of CD61 PerCP, CD62P PE and PAC-1 FITC. The positive control was also incubated with PAC-1 in the presence of Arg-Gly-Asp-Ser (RGDS) to verify specific antibody binding. Samples were fixed with paraformaldehyde 1% for 30 minutes at 4°C in the dark and analyzed on a flow cytometer. *Results*. A higher percentage of CD61+/PAC-1+ events (mean=52.3% *vs* 30.9%) was observed in patients receiving aspirin prophylaxis, indicating increased ADP-mediated platelet activation and a greater expression of PFRs. Statistical significance was reached (p=0.0026). No differences were found with respect to JAK2 Val617Phe mutation. *Conclusions*. Our results now need to be matched with long-term clinical observations to assess the risk of thrombosis in the different groups. Nevertheless, they would seem to indicate an escape mechanism for platelets and a potential role for other antiplatelet drugs in the prevention of vascular events in patients with MPNs.

PO-083

LONG-TERM OUTCOME OF 209 YOUNG PATIENTS WITH ESSENTIAL THROMBOCYTHEMIA

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Introduction. Young adults with Essential Thrombocythemia (ET) or early Primary Myelofibrosis (early-PMF) are a category of patients (pts) projected to a prolonged utilization of medical resources; however, few data are available on their long-term outcome. *Methods*. A clinicopathologic database of ET pts followed in two Italian Hematology Centers was created. A total of 209 WHO-diagnosed ET and early-PMF pts ≤ 40 years at diagnosis was retrieved from the general database of 1323 pts. Baseline clinical/molecular characteristics and outcome measures (vascular complications, disease transformation/progression, overall and event-free survival) were evaluated. *Results.* Overall, 198 WHO-defined ET and 11 early-PMF (age range: 16-40, median 34) were included in the study. Overall, 96 pts carried the JAK2V617F mutation (5 homozygous); in the remaining patients, CALR and MPL mutation were evaluated. No difference were detected between ET and early-PMF pts in terms of baseline clinical characteristics and therapeutic interventions. Median follow-up was 10.2 years (range: 0,5-37,3). 23 and 9 pts experienced a total of 35 thrombotic (arterial: 28.5%) and 14 hemorrhagic events, with an incidence rate of 0.99% and 0.55% pts/yr, respectively. Eleven (5.5%) and 3 (1,5%) ET pts evolved to MF and AL, respectively; 10 developed a second neoplasia (9 ET pts, 4.5%). At last contact, 8 (4%) ET pts had died, 6 for causes related to ET (disease evolution or thrombotic complications). Event-free survival was similar in the ET/early-PMF cohorts considering both every event separately and all together. Consequently, risk factors for each outcome measure were explored only for the ET cohort. Multivariate Cox analysis showed that previous thrombosis, treatment with ≥2 therapies and baseline platelet count <1000x109/L significantly correlated with increased thrombotic risk (p<0.001, p=0.001 and p=0.003, respectively), while splenomegaly at diagnosis predicted higher bleeding risk (p=0.014). Splenomegaly correlated also with greater incidence of secondary MF (p=0.025) together with abnormal karyotype (p=0.031). In univariate analysis, no factor was associated with overall survival. The JAK2V617F mutation did not correlate with any event; however, additional data on impact of CALR mutational status on outcome will be provided. Also, pregnancy outcome will be detailed. *Conclusions*. With the limitations due to the retrospective nature of the study, the limited number of early-PMF cases and the absence of a centralized histological review, the outcome of young adults with early-PMF and true ET seemed to be comparable. The correlation of abnormal karyotype with MF transformation suggests the need for an accurate cytogenetic analysis at diagnosis. Although the number of events was low throughout the follow-up, causes of death were mostly related to the hematological malignancy, pointing out the substantial impact that this generally indolent disease may acquire in young adults.

PO-084

A CALPAIN-CLEAVED FRAGMENT OF β-CATENIN PROMOTES BCR-ABL1+ CELL SURVIVAL EVOKED BY AUTOPHAGY INDUCTION IN RESPONSE TO IMATINIB

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Autophagy protects chronic myeloid leukemia stem cells from tyrosine kinase inhibitors hence supporting the disease persistence under therapy. However, the signals involved in autophagy regulation relative to BCR-ABL1 are still elusive. The autophagic flux proceeding from the inhibition of BCR-ABL1 tyrosine kinase inhibition represents a regulatory mechanism of β-catenin stability through events encompassing the activation of calpain, which targets β-catenin for proteasome-independent degradation. Accordingly, its inactivation may contribute to induce autophagy and autophagy induction may, in turn, promote β-catenin autolysosomal degradation to originate a regulatory loop where β-catenin plays a central role in cell decision between life and death. Here we proved that the cytoplasmic accumulation of β-catenin driven by up-regulation of its antagonist Chibby1 is a component of autophagy induction in response to imatinib in BCR-ABL1+ cells opposing the apoptotic death. Autophagy induction is contingent upon ER stress and elevation of free Ca2+ cytosolic and results in the calpain cleavage into a 28 kDa fragment implicated in β-catenin proteasome-independent degradation. More important for BCR-ABL1+ cell survival and proliferation following IM treatment might be the calpain-mediated cleavage of β-catenin accumulated within the cytoplasmic compartment into a 75 kDa fragment, still owning TCF-dependent transcriptional activity. Such a β-catenin fragment might be crucial for BCR-ABL1+ cell survival following inhibition of the BCR-ABL1 fusion protein tyrosine kinase.

PO-085

MORGANA/CHP1 ACTS AS AN ONCOSUPPRESSOR IN CHRONIC MYELOID LEUKEMIA

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Introduction. Morgana/chp-1 is a ubiquitously expressed CHORD (cysteine and histidine rich domains) containing protein, endowed with chaperone activity. Morgana/chp1 is essential for embryonic development in mouse, since morgana null embryo die early after implantation due to a higher frequency of supernumerary centrosomes, multipolar spindles, aneuploid and polyploid karyotypes. Morgana forms a complex with ROCK I and ROCK II and, by inhibiting ROCK II kinase activity, suppresses centrosome overduplication. Here we show that morgana +/- mice spontaneously develop a lethal myeloproliferative disease resembling human atypical chronic myeloid leukemia (aCML), preceded by ROCK hyperactivation, centrosome amplification and cytogenetic abnormalities. Moreover, we found that morgana is also underexpressed in human atypical CML and in a portion of Philadelphia positive CML (Ph+ CML). *Materials and Methods*. Conventional flow cytometry analyses, colony forming assays and disease transplantation assays have been performed to characterize the hematological disorder observed in morgana +/- mice. Human samples of aCML and Philadelphia positive CML have been assayed for the expression of morgana by immunohystochemistry, flow cytometry and western immunoblot. Primary cells were also treated with ROCK inhibitors and apoptosis was evaluated by flow cytometry. *Results*. Morgana +/- mice developed with age a fatal MPD disorder characterized by splenomegaly and a significant increase of Mac-1+ Gr-1+ and Mac-1+ c-Kit– cells in the spleen and in the bone marrow. In few cases, murine MPD progressed toward accelerated/blast phase. Murine MPD was transplantable into nude mice. Notably, in morgana +/- MPD we observed increased ROCK activity, centrosome amplification and aneuploidy. To assess whether morgana/chp1 is expressed in human MPD, we performed immunohistochemistry on 5 aCML patients and on 26 Philadelphia positive CML. Strikingly, we observed that in all aCML patients morgana is dramatically underexpressed and correlates with increased ROCK activity. Furthermore, 16% of Philadelphia positive CML expressed low levels of morgana when assessed by immunohistochemistry. Notably, Morgana underexpressing CML patients are characterized by suboptimal response to Imatinib treatment. Finally, we observed that treatment with Rock inhibitor Fasudil induces apoptosis of morgana underexpressing CML cells. *Conclusions*. Morgana/chp1 acts as an oncosuppressor with different modalities: on one hand, morgana underexpression induces centrosome amplification and cytogenetic abnormalities, on the other, in Ph+ CML, it synergizes with BCR-ABL signaling, reducing the efficacy of Imatinib treatment. Importantly, ROCK inhibition in CML underexpressing morgana restored the efficacy of Imatinib to induce apoptosis, suggesting that ROCK inhibitors, in combination with Imatinib treatment, can overcome suboptimal responses in patients in which morgana is underexpressed.

PO-086

QUANTIFICATION IN RT-PCR OF IMATINIB TRANSPORTER MAY NOT HAVE A PROGNOSTIC ROLE IN PREDICTING THERAPY REFRACTORINESS IN CHRONIC MYELOID LEUKEMIA WITH WYLDE-TYPE BCR-ABL

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Background and Aim. Imatinib mesylate has dramatically improved the life expectancy of many patients affected by chronic myeloid leukemia (CML). However a small proportion of patients do not benefit from this therapy while most patients with CML in accelerated phase or in blastic crisis do not respond to Imatinib for the presence of various mechanism of drug resistance. Using Q-RT-PCR we measured the levels of expression of hOCT1 (the only carrier for the uptake of Imatinib) and of the main Multi Drug Resistance proteins (ABCB1, ABCC1, ABCC3, ABCG2) which are responsible for the output of the drug from the leukemic cell. *Materials and Methods*. The physiologic levels of the carrier-proteins were determined from leukocytes' RNA of peripheral blood from 50 healthy subjects (25 male, 25 female). Then 33 patients were retrospectively analyzed with the aim to verify if there may be a correlation between the transporter expression and imatinib resistance; so we proceeded to verify if the achievement of a major molecular response after six months of therapy with imatinib could correlate with the expression of the carriers at the onset of CML. *Results*. The data obtained by Q-RT-PCR showed that the expression levels of transporters for imatinib mesylate are variable in patients with CML, in a relatively narrow range even though more variable than in healthy subjects. In the 33 patients analyzed retrospectively there are an extreme variability in the values of expression. It was performed a matrix of Pearson correlation, which showed a lack of correlation between the expression of the five genes (hOCT1, ABCC1, ABCC3, ABCB1, ABCG2) and the response after 6 months of imatinib therapy. However, there is a non significant trend (probably due to the sample analyzed) toward a higher response rate in patients overexpressing hOCT1, regardless of the expression of the other channels (X-squared=0.1393, df=1, p-value=0.709). It is likely that our study was not large enough to obtain a significant value in univariate (Figure 1). *Conclusions*. This study showed that the levels of hOCT1 and MDR proteins (ABCB1 , ABCC1 , ABCC3 , ABCG2) are variable in patients with CML, with a relatively narrow interquartile range, although higher than in healthy subjects. Some of the trans-membrane channels tend to be co-expressed: ABCC3 with ABCB1 , ABCC1 with ABCG2, hOCT1 with ABCB1. Statistical analysis demonstrated that the expression of these genes is not significantly correlated with the response at 6 months; however, there is a non significant trend toward a higher frequency of response in patients overexpressing hOCT1, regardless of the expression of the other channels . Based on the observed data it does not seem possible to predict the response to 6 months imatinib treatment from the expression of these channels.

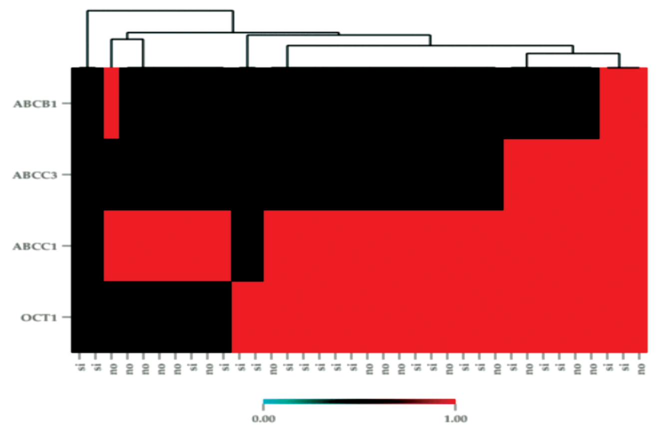

Figure 1. Abscence of dichotomisation between the patients which are over-expressing or not the transmembrane transporters

PO-087

ULTRA-DEEP SEQUENCING ALLOWS MORE SENSITIVE DETECTION OF THE D816V AND OTHER KIT GENE MUTATIONS IN SYSTEMIC MASTOCYTOSIS

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Introduction. Systemic Mastocytosis (SM) is a clonal disease characterized by abnormal growth and accumulation of mast cells (MC) in one or more organs. The great majority of patients (90%) showed the somatic ‚Äòautoactivating' point mutation D816V in the KIT receptor gene which leads to constitutive phosphorylation of KIT and consequent activation of downstream molecules. The D816V mutation play a central role in the pathogenesis, diagnosis, and therapy of SM and is considered as one of the minor criteria for its diagnosis by the 2008 World Health Organization (WHO). Indolent Systemic Mastocytosis (ISM), the most common variant of SM, is a rare entity to diagnose characterized by a very low MC burden; in these cases highly sensitive diagnostic methods for D816V

detection are required to assure an appropriate diagnosis and to reduce false-negative results. The recent development of "ultra-deep amplicon sequencing" (UDS) technologies has opened the way to a more accurate characterization of molecular aberrations with higher sensitivity of screening for known and unknown mutations. Our aims were: i) to set-up and optimize a UDS-based mutation screening strategy of the KIT gene on the Roche GS Junior Instrument; ii) to test the sensitivity of our UDS assay to detect the D816V mutation; iii) to investigate the presence of additional KIT mutations in SM. *Methods.* Fusion primers were designed to generate ten partially overlapping amplicons covering the whole KIT transcript by RT-PCR (exons 1-21). To determine the lower detection limit of our UDS-assay, serial dilutions of the HMC-1 cell line (harboring the D816V mutation) into an unmutated K562 cell line in ratios such as to simulate the following mutation loads were sequenced: 50%, 37.5%, 25%, 12.5%, 5%; 2.5%, 1.25%, 0.5%, 0.25%. UDS KIT gene mutation analysis was performed on 4 bone marrow (BM) samples obtained from patients whit ISM already studied by SS. *Results*. Our results showed a high accuracy of D816V mutation detection and linearity of mutation calling and over the entire range of dilutions, down to 0.25%. Interestingly, NGS allowed to detected the D816V mutation, with an abundance from 1% up to 5%, in 3/4 ISM patients who were negative by SS. UDS also revealed a 3bp in-frame deletion in exon 15 (c.2164_2166delAGC; p.S715del) in 4 patients. The COSMIC database revealed that this variant has previously been found in gastrointestinal stromal tumors but not in SM. In addition, UDS detected a novel 12bp in frame-deletion in exon 9 in 4 patients, whit an abundance ranging from 83% to 94% (c.1550_1561delGTAACAACAAAG; p.G510_K513del). Analysis of 50 additional cases is ongoing. *Conclusions*. Our preliminary results underline that our-UDS based KIT gene mutation screening assay might be a reliable and sensitive alternative to conventional sequencing methods for the detection of the D816V and for the identification of new mutations/sequence variations associated with SM.

Chronic Lymphocytic Leukemia and Chronic Lymphoproliferative Disorders II

PO-088

RESVERATROL MONO-METHOXY DERIVATIVE INDUCES APOPTOSIS OF LYMPHOMA CELLS THROUGH MODULATION OF p53, SIRT1 AND CASPASE-3

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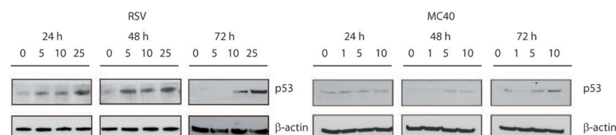
Introduction. Diffuse large B cell lymphoma (DLBCL) accounts for approximately 30% of the Non-Hodgkin lymphomas (NHL). The classification of DLBCLs into Germinal Center like (GCB) and Activated B cell like (ABC) is based on their different gene expression pro-files and their relevance for the clinical prognosis. The phytochemical Resveratrol (RSV) has been shown to exert numerous health benefits, but its rapid metabolism and poor bioavailabil-ity limit the translation of these effects to humans. Analysis of structure–activity relationship reveals that the substitution of hydroxyl to methoxygroups potentiated RSV cytotoxic activity. Therefore, some methoxylated analogs have been designed with the aim of increasing the antitumor activity of RSV. *Methods.* Trans-4'-hydroxy-4-methoxystilbene (MC40); trans-4'-hydroxy-3,5-dimethoxystilbene (MC43); trans-3,4'-dimethoxysilbene (MC149) were synthesized at Uni-versity of Burgundy, France. Cell viability, IC50 calculation and apoptosis. Colony forming assay on methylcellulose. Confocal Microscopy. Western blot. Enzymatic activity. Cell lines: GC (Toledo) and ABC-DLBCL (OCILy3). *Results.* Our results shows that two derivatives (MC40 and MC43) are more powerful than RSV. MC40 shows the strongest activity with an IC50 of 0.9 µM and 3.9 µM on Toledo and OCILy3 cell lines respectively (13 and 4.5 fold better than RSV). MC40 displays marked pro-apoptotic properties and induces the: i) up-regulation and nuclear localization of p53, ii) dose-dependent activation of caspase-3 and iii) down-regulation of the histone/lysine deacetylase SIRT1 at the highest concentrations tested. The colony forming potential is inhibited by both RSV and MC40. Interestingly, the growth inhibition caused by RSV and MC40 is diminished after the treatment with a specific caspase-3 inhibitor in Toledo cells whereas the apoptosis observed in OCI-Ly3 cells is caspase-3 independent. Fi-nally, RSV up-regulates the autophagic marker LC3 II in OCILy3, suggesting that this mechanism may contribute to the observed caspase-3 independent cell death (Figure 1).



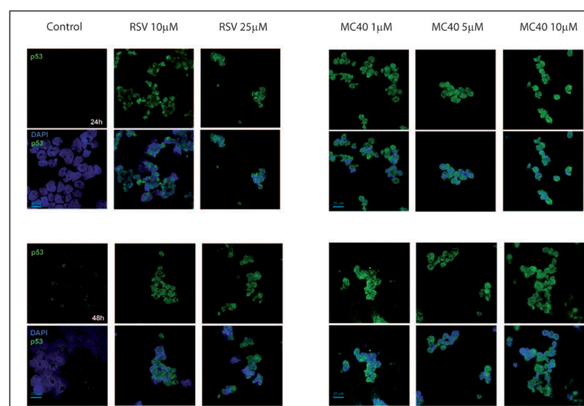


Figure 1.

Conclusions. These data show the *in vitro* characterization of the antiproliferative and pro-apoptotic properties of the mono-methoxy RSV derivative MC40 in lymphoma models. Our experiments support the involvement of p53, SIRT1 and caspase3 during RSV- and MC40-mediated apoptosis of NHL cells. These data prompt us for future *in vivo* challenges.

PO-089

NULL PHENOTYPE WITH UNIQUE HALLMARK: ROLE OF IMMUNOCYTOCHEMISTRY

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Introduction. Peripheral blood (PB) involvement in anaplastic large cell lymphoma (ALCL) is uncommon, either at disease onset or during clinical course. To our knowledge, only about 20 cases have been reported in the literature. However, this rare entity shows unique phenotypic and genetic hallmarks, which can be detected by immunocytochemistry on PB smears. *Methods*. A 66-year old man without previous medical history was observed in October 2013, because of the finding of left axillary lymph node enlargement on ultrasonography, performed owing to persistent shoulder pain. During the last week before presentation, he became febrile and complained progressive fatigue, hyporexia and generalized malaise. On admission, blood counts showed severe thrombocytopenia, moderate normochromic normocytic anemia and moderate leukocytosis (WBC 17x10^9/L, Hb 9.5 g/dl, MCV 84 fl, PLT 30x10^9/L). Lactic dehydrogenase was moderately increased and renal function was slightly impaired (serum creatinine 1.7 mg/dl, GFR 39 ml/min). *Results*. PB smear documented the presence of 37% atypical cells, heterogeneous in size (Figure 1).

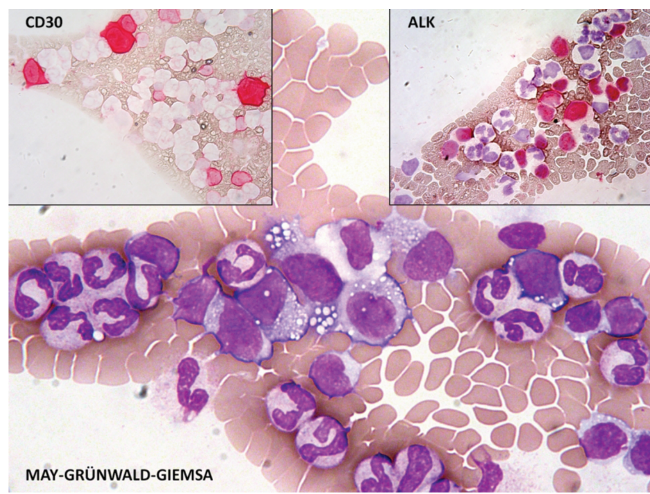

Figure 1.

The small/medium-sized cells showed high nuclear-cytoplasmic ratio with scant cytoplasm and irregular cerebriform, sometimes cloverleaf-shaped nuclei, with condensed chromatin and inconspicuous nucleoli. Conversely, large cells displayed abundant deeply basophilic, frequently vacuolated cytoplasm and moderately condensed chromatin, rarely horseshoe-shaped nuclei. Therefore, an aggressive hematologic malignancy of ambiguous lineage, due to the wide spectrum of PB morphologic abnormalities, was suspected. Flow cytometry investigation on PB sample documented a pathologic population (41% of the events) with medium side scatter and high CD45 expression, attributable to lymphocyte gate. Immaturity (CD34, Tdt) and lineage specific antigens (CD19, CD3, myeloperoxidase, CD64) were negative. Based upon morphologic features and "null" phenotype of atypical cells, immunocytochemistry on PB smears was performed, documenting overt CD30 (left inset) and anaplastic lymphoma kinase (ALK) (right inset) positivities. FISH analysis subsequently demonstrated ALK rearrangement (2p23 break-apart probe) on 19% PB cells. BM aspirate and trephine biopsy confirmed PB findings. Final diagnosis was leukemic phase of ALCL, ALK-positive, according to WHO Classification 2008. The patient underwent chemotherapy according to cyclophosphamide-doxorubicin-vincristine-etoposide-prednisone (CHOEP) regimen. *Conclusions*. Immunocytochemistry remains a useful, sensitive, cost-effective and rapid diagnostic tool, which may be crucial in cases of severe clinical presentation of hematologic disorders. As a matter of fact, this method permitted, in our case, an early diagnosis, before completion of BM immunohistochemical examinations and genetic studies.

PO-090

REGULATORY T-CELLS DETECTABLE IN PERIPHERAL BLOOD IN HODGKIN'S LYMPHOMA INCREASE DURING ABVD CHEMOTHERAPY, EXCEPT FOR PATIENTS WITH POSITIVE INTERIM PET

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Introduction. Recent observations suggest a prognostic role for regulatory T cells (Treg) in classical Hodgkin lymphoma (HL) tissues, correlate with poor overall survival both at diagnosis and in relapsed/refractory cases. Currently, no data have been published in the era of interim positive emission tomography PET (PET-2)- risk adapted strategy. *Objectives*. To investigate whether the frequency of peripheral blood (PB) regulatory T cells (Treg) correlates with the clinical disease activity of HL. *Methods.* PB Treg cells, defined as the CD4+CD25+(high)CD127(low/-) population, were examined by flow cytometry in a prospective study involving 20 healthy donors and 25 patients with newly diagnosed HL, including 15 in early stage and 10 with advanced disease at different timepoints (T0=baseline, T2 after two courses of ABVD chemotherapy, T4, after two months from the last course of chemotherapy). The association of C-reactive protein (CRP), fibrinogen level or erythrocyte sedimentation rate (ESR) with the frequency of PB Treg cells was examined. Baseline levels were correlated with the ability to predict patients with a positive PET-2. *Results.* The frequency of PB Treg cells in patients with HL was significantly low compared with that of healthy controls in terms of percentage (mean ±DS, 5.9%±2.1 *vs* 8.5%±2.1, p=0.0004) and absolute numbers (mean ±DS, 30.3±16.4 cells/uL *vs* 75.5±25.6 cells/uL, p<0.0001). Among the 3 timepoints tested, Treg cell frequency was lowest in patients at diagnosis with a progressive increase during chemotherapy (p=0.0028). Patients with positive PET-2 exhibited lower count of Treg compared to patients with a negative PET-2 at diagnosis (mean ±DS, 12.5±7.2 cells/uL *vs* 33.1±16.1 cells/uL, p=0.0013) but no differences were due to presence of bulky disease or stage at diagnosis. The absolute count of Treg cells weakly negatively correlated with CRP and ESR, but not with fibrinogen. *Conclusions*. Taken together our data suggest that Treg cells, defined as the CD4+CD25+(high)CD127(low/-) population, may contribute to the pathogenesis of HL and be an indicator of disease activity, easily detectable in PB, to be confirmed prospectively in a larger series.

PO-091

ARGINASE-1 IS AN IMMUNOSUPPRESSOR MEDIATOR INCREASED IN HODGKIN'S LYMPHOMA NEUTROPHILS ASSOCIATED TO POOR OUTCOME

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Introduction. In Hodgkin Lymphoma (HL) elevated neutrophil (HL-N) count is a well recognized negative prognostic factor but its biological meaning is not elucidated. Our previous work showed that HL-N are dysfunctional and can suppress T-cell activation *in vitro*. Arginase (Arg-1) in blood cells is mainly stored in N-granules and released as inflammation suppressor. In order to investigate immunosuppressive properties of HL-N we evaluated arginase expression and activity in HL-N. *Materials and Methods.* In N obtained from 15 HL patients we tested phagocytic activity by flow-cytometry, enzymatic activity of Arg-1 by colorimetric method, expression of Arg-1 in RT-PCR, and suppression of healthy T-lymphocytes activation in co-cul-

ture experiments. Amount of Arg+ cells was also evaluated in HL lymphonodes by IHC. We prospectively measured soluble Arg-1 (s-Arg-1) in 135 sera obtained from 60 patients with HL, distinguished in a training set (N=25) and a validation set (N=35) and 21 healthy participants. In the training set, blood was taken at three fixed time-points prior, during, and after first-line therapy. Findings were compared with radiological assessment, including 2FDG-positron emission tomography scan after 2 cycle of chemotherapy (PET-2) and clinical variables. *Results.* N-HL exhibited a reduced phagocytosis (93.2 ±1.9 % *vs* 73.1±3.7, p=0.0008) and an increased arginase activity up to 15 times compared to healthy subjects matched for age and sex. We observed an increase of Arg-1 expression in HL-N up to 100 folds compared to healthy subjects matched for age and sex (p=0.001), independently from tumor load and other well-known prognostic factors, including sex, anemia, stage, bulky disease and IPS. In the lymphonodes, Arg-1 evaluated in immunohistochemistry showed a granular pattern distribution in lack of overlapping with CD68+ staining.s-Arg-1 was increased in HL patients compared to healthy subjects, reduced after therapy in responders and increased in relapsed patients (p<0.0001). s-Arg-1 was positively correlated to the amount of N and Arg-1 in N detected by RT-PCR. A cut-off level of 205 ng/mL for Arg-1 was chosen (equal to 2 times the 95th percentile in controls and ROC value with sensitivity and specificity of at least 80%) to predict response status at 24 months. Patients with s-Arg-1 ≥ 205 ng/mL had shorter PFS than patients carrying Arg-1 < 205 ng/mL. *Conclusions.* Neutrophils in HL are dysfunctional for high amount of Arg-1. S-Arg-1 is a predictor of PFS even in the cohort of advanced-stage patients early addressed to salvage regimen in case of PET-2 positivity.

PO-092

GENE-EXPRESSION PROFILE OF GRANULOCYTES AT THE STEADY-STATE IN HODGKIN LYMPHOMA

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Introduction. The role of microenvironment in the pathogenesis of Hodgkin's Lymphoma (HL) is well recognized. Our previous work showed that myeloid derived suppressor cells are increased in newly diagnosed HL patients and have a prognostic significance. *Aim*. Providing a detailed analysis of granulocyte function at the steady-state in HL at baseline and after two cycles of therapy in unresponsive patients. *Methods*. Using oligonucleotide microarrays we first evaluated the gene expression profile of granulocytes at the steady state in 4 HL (at baseline and after therapy) and 3 healthy subjects matched for sex and age. Then, we validated preliminary findings in granulocytes and serum obtained from peripheral blood in 40 consecutive newly diagnosed HL and 15 healthy subjects. We measured the following surrogates of activation/degranulation/myeloid suppression: i) soluble markers of inflammation: s-Arg1, TNF-α, s-IL-2-R using commercially available ELISA kits; ii) Arg-1 and BV8 in RT-PCR. *Results*. We found 577 genes differentially expressed (297 up- and 280 down regulated) in HL at baseline *versus* healthy granulocytes at the steady state, and 210 genes differentially expressed (111 up- and 99 down regulated) between baseline and post-therapy evaluation in patients unresponsive to treatment. In particular, we identified eight genes up-regulated in N-HL involved in T-cell immunosuppression: ARG-1, PROK-2, TLR-5, IL17RA, IFNRA1, IL4R, CD16, CD14 (p<0.0001). In unresponsive patients after therapy ARG-1, IL17RA and CD16 were further increased compared to baseline (respectively p=.002, p=.0006, p=.009). In an independent set we confirmed by RT-PCR that HL at baseline had higher expression of ARG-1 and BV8 (respectively 25 and 12 times, p<0.0001). Circulating Arg-1 in serum (s-Arg-1) was increased in HL patients compared to healthy volunteers, (186 *versus* 58 ng/mL, p=0.002), similarly to TNF-α (23.5 *versus* 12.6 pg/mL, p=0.013) and s-IL-2-R (2.9 *versus* 1.3 ng/mL, p=0.012). *Conclusions*. Granulocytic impairment is present in HL patients at the steady state due to increased expression of genes that negatively regulate adaptive immune response. Further studies will identify those could contribute to immunological paresis mediating resistance to chemotherapy.

PO-093

TRANSCRIPTION FACTOR EXPRESSION IN PERIPHERAL BLOOD T CELLS IN HODGKIN LYMPHOMA: ASSOCIATIONS WITH DISEASE CHARACTERISTICS

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Introduction. In Hodgkin lymphoma (HL), the tumor microenvironment plays an important role in supporting the proliferation of the neoplastic cells and in propagation of this disease. The T cell population in the microenvironment is characterized by an expansion of TH2 and Treg cells, that are probably attracted and expanded by chemokines and cytokines produced by the Reed–Sternberg (RS) cells. *Aims.* We hypothesized that the T cell population in the peripheral blood may reflect the functional changes of the tumor-infiltrating T cells in HL. In a first step, we focused on characterization of the functional state of T cells in peripheral blood to look for systemic changes in T cell function beyond the local microenvironment. *Methods.* T cells were isolated from peripheral blood of 52 HL patients (24 males, 28 females; median age 38 years, range 16-74) at diagnosis and 11 healthy controls using density gradient centrifugation followed by magnetic beads separation with the PanT cells isolation kit (Mylteni). The functional state of the T cell compartment was studied by mRNA expression of genes in Real Time PCR that indicate commitment of T cells toward a Th1 (T-BET), Th2 (GATA3), or T-reg (FOXP3) phenotype. P105 and IKBA expression were used as indicators of activation and inhibition of the NFkB pathway, respectively. To evaluate the prevalent commitment of peripheral T cell population we calculated the ratios between GATA3/T-Bet and FOXP3/T-BET. *Results*. Patients with HL (n=52) had significantly lower expression levels of transcription factors GATA3 (p=0.0006), FOXP3 (P=0.001), T-BET (P=0.003) and P105 (P=0.021) in peripheral blood T cells when compared to controls (n=11). No difference was observed for IKBA expression levels, and ratios of GATA3/T-BET and FOXP3/T-BET were maintained. Advanced stage disease was associated with reduced expression levels of FOXP3 and T-BET in peripheral blood T cells (p=0.02 and p=0.007). As well, an IPS score >2 was associated with reduced FOXP3 and T-BET expression levels (both p=0.02). An interesting association became evident when analyzing for correlations between peripheral blood cellular subsets and T cell transcription factors. The monocyte count inversely correlated with T cell expression levels of GATA3 (rs=-0.49, p=0.0004), FOXP3 (rs=-0.46, p=0.001), and T-BET (rs=-0.42, p=0.0025). Similarly, a proportion of CD68+ cells in the tumor tissue >5% was associated with lower levels of GATA3 (P=0.025), T-BET (0.018), P105 (P=0.015) and IKBA (0.041). *Conclusions*. Our study shows a reduced expression of transcription factors indicative for TH1, TH2 and Treg commitment and activation in peripheral blood T cells in patients with HL. Further studies are needed to clarify whether the inverse correlation of the T cell transcription factors to the monocyte compartment in peripheral blood and the tumor microenvironment may be due to a functional cross-talk between T cells and monocytes.

PO-094

TARGETING ADAM10 SHEDDASE WITH SPECIFIC INHIBITORS IN HODGKIN LYMPHOMA

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Stress-related immunity contributes to anti-neoplastic surveillance through the activation on effector T lymphocytes of NKG2D that recognizes on cancer cells NKG2D-ligands (NKG2D-L), including MHC class-I related chain –A and –B (MIC-A/B) and UL16-binding proteins 1-4 (ULBPs). Expression of these ligands is enhanced in neoplastic cells and can be up-regulated by anti-leukemic drugs, as all-trans-retinoic acid or sodium valproate (VPA). We described that a subset of γδ T lymphocytes is expanded in patients with non-Hodgkin lymphomas (NHL) and proliferate in response to tumor cells, provided they express NKG2D-L, exerting cytotoxicity and producing anti-neoplastic

cytokines. However, NKG2D-L can be shed, interact with NKG2D expressed by effector lymphocytes and hinder tumor cell recognition. Proteolytic cleavage of NKG2D-L can depend on the disintegrin-and-metalloproteinases ADAM10/ADAM17, that are overexpressed in several tumors. Moreover, we and others reported that plasma levels of sNKG2D-L correlate with disease progression in multiple myeloma, CLL, NHL and acute myeloid leukemias. Herein we show that in Hodgkin lymphoma (HL), both lymph node mesenchymal stromal cells (LNMSC) and Reed-Sternberg (RS) cells displayed *in situ* high levels expression of ADAM10; also, MIC-A and ULBP3 were present in culture supernatants of LNMSC or RS cells. Upon co-culture with LNMSC, T cells strongly reduced their cytolytic activity against NKG2D-L+ targets, due to TGFβ, present at the tumor site, produced *in vitro* by LNMSC and able to down-regulate NKG2D expression. We have synthesized ADAM10/ADAM17 inhibitors in order to prevent sNKG2D-L release by LNMSC or RS cells: results of experiments with siRNA point to a predominant role of ADAM10 *vs* ADAM17 in RS cells and at least one specific ADAM10 inhibitor has been shown to interfere with the sheddase activity. In conclusion, in HL 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: prevention of NKG2D-L release, through the inhibition ADAM10 enzymatic activity can be proposed as potential therapeutic targets.

PO-095

THE HISTONE DEACETYLASE INHIBITOR ROMIDEPSIN SYNERGIZES WITH LENALIDOMIDE ENHANCING TUMOR-CELL DEATH IN T-CELL LYMPHOMA CELL LINES

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Introduction. Romidepsin is a potent and specific inhibitor of class I and II HDAC inhibitor that has shown remarkable activity in the treatment of T-Cell Lymphoma (TCL) in preclinical studies and early-phase clinical trials. Lenalidomide is an immunomodulatory agent highly effective for the treatment of a wide range of hematological malignancies, and a low toxicity profile. Our goal is to evaluate if lenalidomide, in combination with romidepsin is able to enhance the apoptotic effect on TCL cell lines and to identify the molecular mechanisms involved. *Methods.* Hut-78 and Karpas-299 were treated with increasing concentrations of romidepsin (0,5-25 nM) and lenalidomide (1-100 µM) alone from 24-72h to identify the IC50 of each drug. The interaction between romidepsin (0,5nM, 1nM, 2,5nM) and lenalidomide (2µM, 4µM, 10µM) was evaluated using the Chou-Talalay method. The cell cytotoxicity was assessed by MTT assay and apoptosis was studied by Annexin-V/propidium iodide (PI) and flow cytometry and confirmed by Western blot analysis for caspase activation. AKT/PI3K and MAPK/ERK signaling pathways were analyzed by Western blot. *Results.* Treatment with romidepsin alone resulted in time- and dose-dependent increase in cytotoxicity in Hut-78 and Karpas-299 cell lines with an IC50 at 24h of 5.87 nM and 6.36 nM for Hut-78 and Karpas-299, respectively. Lenalidomide alone did not inhibit cells viability up to72 h of treatment in the two TCL cell lines. The combination of lenalidomide (10µM) with a low dose of romidepsin (2.5 nM) showed a strong synergistic interaction with combination index (CI) of 0.14 in Hut-78 cells and an additive effect in Karpas-299 cells, (CI of 1.08) after 24h. In Hut-78 cells sequential treatment with romidespin for 6h followed by washout and the next addition of lenalidomide for 24h enhanced the cytotoxic effect of romidepsin and confirm its irreversible effect. The combination did not trigger relevant decrease in the viability of normal peripheral blood mononuclear cells (PBMNCs). Romidepsin and lenalidomide in combination induced apoptosis with little effect on cell cycle. Interestingly, the treatment with the caspase inhibitor z-VAD-fmk allowed detection of cell cycle arrest in G0/G1 phase. The apoptotic effect of the combination was confirmed by the activation of caspases -3, -9 -8 and PARP and was mediated by Bim, Bax, Bad, Bcl-xL and Mcl-1. The drugs combination did not modify Bcl-2 expression. The drugs combination induced up-regulation of cell cycle protein p21, p27 and a slight decrease of cyclin E and cyclin D. These events were associated with the dephosphorylation of PI3K/Akt, MAPK/ERK pathways. Induction of histone acetylation (H3) and acetylated alpha-tubulin was confirmed. *Conclusions*. The combination of romidepsin with lenalidomide induces synergistic anti-proliferative and pro-apoptotic effects in TCL through signaling event involving the pro-survival pathways PI3K/AKT and MAPK/ERK.

PO-096

INHIBITION OF AUTOPHAGY ENHANCES APOPTOSIS INDUCED BY THE NVP-BEZ235 AND BKM-120 IN LYMPHOID MALIGNANCIES

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BKM120 and BEZ235 are synthetic small molecules belonging to the class of imidazo-quinolones that show preclinical activity against a range of solid and hematological malignancies. BKM-120 inhibits the catalytic subunit of class I PI3K by competitive binding to its ATP binding site, while BEZ235 is a dual class I PI3K/mTOR inhibitor. The PI3K/Akt/mTOR signaling pathway regulates several normal cellular functions that are critical for tumorigenesis, including cellular proliferation, growth, survival and motility. The PI3K/AKT/mTOR signaling pathway also inhibits cell autophagy, a catabolic process involving the degradation of a cell's own components through the lysosomal machinery. In this study we investigated the effects of BKM120 and BEZ235 on survival rate, apoptosis, signaling pathways expression and autophagy in lymphoma cell lines. *Methods*. Lymphoma cell lines were treated with different concentrations of BKM120 and BEZ235 (Novartis) and the IC50 values were evaluated using MTT assay. To assess for apoptosis, we used annexin V/PI staining kit/ flow cytometer analysis and Western Blot to evaluate caspase 3, 8, 9 and PARP expression. The cell cycle was performed applying PI incorporation and flow cytometer analysis. Quantitative analysis of the levels of alpha tubuline was done with flow cytometer to test whether the two drugs could cause a mitotic block. Western blot was utilized for phosphorilation status of protein kinases and for monitoring autophagy. *Results*. BKM120 and BEZ235 induced significant increase of apoptosis evidenced by annexin IV/PI staining and confirmed by the cleavage of caspases -3, -9 -8 and PARP. BKM120 and BEZ235 induced an upregulation of proapoptotic protein Bim, Bax and Bad. Treatment for 24h with BKM120 and BEZ235 resulted in different effects on cell cycle. BKM120 induced an increase of G2-phase with down regulation of Cyclin D and E, and an up-regulation of Cyclin A, p21 and p27. The increase in G2-M caused by BKM120 treatment occurred in a dose dependent manner. BEZ235 induced an increase of Go/G1-phase with up regulation of Cyclin A, D, E and p21 and p27. Quantitative analysis of gamma and alpha tubulins staining of the cell lines revealed that treatment with BKM120 induced an accumulation of mitotic cells. BKM120 and BEZ235 are inhibitors of intracellular pathways in targeting p-Akt, p- mTOR, pS6K, 4EBP1, MYC and STAT. BKM120 and BEZ235 increased the levels of type II LC3 and p62, hallmarks of autophagy, in addition to increasing caspase 3 cleavage and annexin positive cells, suggesting that the two drugs induced both apoptosis and autophagy. The combination of BKM120 and BEZ235 with chloroquine enhanced apoptosis. We concluded that BKM120 and BEZ235 inhibit PI3K/AKT and mTOR signaling and induce autophagy and BKM120 can act as a microtubule destabilizer, highlighting novel mechanisms accounting for the anticancer activity of the two drugs and a potential strategy to enhance the anticancer efficacy.

PO-097

LONG-TERM DYNAMICS OF PERIPHERAL BLOOD NATURAL KILLER CELL IN DIFFUSE-LARGE-B-CELL LYMPHOMA PATIENTS BEFORE AND AFTER RITUXIMAB-CHOP IMMUNOCHEMOTHERAPY

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Background. NK-cells through an antibody-dependent cellular cytotoxicity (ADCC) mechanism might significantly contribute to the suc-

cess of rituximab-based therapies. *Aims*. We focused our work on monitoring the phenotypic and functional asset of peripheral blood NK cell subsets in newly diagnosed Diffuse-large-B-cell lymphoma (DLBCL) and to evaluate its acute and long-term modifications. *Methods.* PBMC from 32 DLBCL patients and 27 healthy, age- and sex-matched controls were analyzed for: 1) the percentage (over PBMC) of CD56dim, CD56bright, and CD16+ NK cell subsets, measured by multi-parameter flow cytometric (FACS) analysis; 2) the functional capability of NK cell subsets: *i.e.* the frequency of IFN γ-expressing cells and cytotoxic granule-containing (granzyme B+, GrzB+) cells, evaluated by intracellular staining and FACS analysis; and 3) "natural" and CD16-dependent NK cytotoxic functions, quantified by 51Cr release assay. Patients' PBMC were analyzed at diagnosis, at mid-therapy, and at different time points up to 12 months after R-CHOP therapy. *Results*. NK cell in DLBCL patients at diagnosis were phenotypically and functionally altered, in comparison to healthy controls: 1) patients' PBMC showed an absolute NK cell count not different from controls, while the percentage of CD56dim and CD16+ NK cell subsets were higher than controls; 2) the frequency of GrzB+ cells was markedly increased in CD56dim, CD56bright, and CD16+ NK cell subsets; 3) "natural" and CD16-dependent NK cytotoxic activities, as well as the percentage of IFN γ-producing NK cells upon *in vitro* stimulation were not different compared to healthy subjects. At mid-treatment, 1) the frequency of CD16+ NK cells 2) the percentage of NKG2D+ cells in CD16+ and CD56 dim NK subsets, and 3) "natural" and CD16-dependent NK cytotoxic activities were significantly reduced. These impairments persisted up to 1 month after R-CHOP, and recovered stably by 3 months after the end of treatment. At odds with the above results, 1) the percentage of GrzB+ cells remained elevated in CD56dim, CD56bright, and CD16+ NK subsets till 3 months after therapy, and returned to normal by 6 months after therapy, and 2) the frequency of IFN γ+ NK cells did not show any significant variation, at any time point. *Conclusions*. 1) The systemic NK compartment at disease onset showed a phenotypic and functional disturbance, resembling a chronic activation state; 2) The marked therapy-induced reduction of "natural" and CD16-dependent NK cytotoxic activities was accompanied by the down modulation of CD16 and NKG2D activating receptors, and was not associated with a gross impairment of NK cell lytic potential and IFN γ production. This suggests that the therapy-driven continuous stimulation through CD16 receptor could be involved in a prolonged NK cell functional impairment *in vivo*. Our observations may be relevant for the improvement of therapeutic strategies in DLBCL. M.C. Cox and S. Battella equally contributed to the study.

PO-098

INCIDENCE OF MULTIPLE MYELOMA FOLLOWING THERAPY FOR NON-HODGKIN AND HODGKIN LYMPHOMA

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Introduction. Multiple Myeloma (MM), non Hodgkin (NHL) and Hodgkin (HL) Lymphoma are lymphoproliferative diseases (LD) arising from B-cells at several differentiation stages. Although previous studies have reported an elevated risk of second malignancies following these disorders, few studies have investigated the development of two LD in the same patient. *Aims.* We assess long-term risk of MM emergence as a second malignancy in patients with NHL and HL. *Methods.* From 2003 to 2013 a cohort of 419 NHL and 277 HL patients, at first diagnosis, were observed from a single center Institution and occurrence of MM was pointed out. Standardized Incidence Ratio (SIR) was calculated as the ratio of observed to expected number of incident MM. *Results.* In total only two and one second MM occurred among NHL and HL patients, respectively. The cumulative risk of developing a MM following NHL and HL was respectively 0.43% (1/230) and 0.36 (1/277) among males and about 0.53% (1/189) and 0% (0/140) among females. SIR for male and female NHL patients was 0.83 (p=0.56) and 1.46 (p=0.79). Therefore, in HL group male and female SIR values were respectively, 0 (p=0.39) and 2 (p=0.61). Among patients with secondary MM, one patient had a previous diagnosis of DLBCL and was treated with R-CHOP scheme for six cycles, the second patient was diagnosed with gastric MALT and was treated with four CHOP cycles and the last one, affected by classical HL, received six courses of ABVD followed by IF radiotherapy. All three patients obtained CR after first line treatment for lymphoma and developed MM after the recognition of MGUS. One of them showed M-component since the time of LNH diagnosis while the other two patients developed it during the follow-up. The average time from first LD and MM diagnosis was 7.6 years (range 5-13), while the average time from MGUS and MM transformation was 3.3 years (range 2-5). Information for light-chain restriction of NHL and MM was available only for the patient with previous MALT diagnosis and it was different in that case. Among these three patients who developed MM, one is on PR after only three course of liposomal doxorucin, bortezomib and dexamethasone, one is on CR after thalidomide plus dexamethasone followed by autologous stem cell transplantation. For the last patient receiving only one administration of bortezomib, thalidomide and dexamethasone, we can't evaluate response. *Conclusions.* The occurrence of MM and LD in the same patient is very rare. It is generally accepted that normal plasma cells are terminally differentiated, specialized cells arising from B-cells, and it is theoretically possible that the monotypic plasma cells are further differentiated or transformed neoplastic B-cells. But the two processes may arise independently from the same stem cell or from different B cells purely coincidentally. Although it would be rare, a possibility of independent of two infrequent malignancies in the same patient exists and seems to be more convincing theory.

PO-099

EMZL SUBTYPE IS ASSOCIATED WITH INCREASED BASELINE PLATELET COUNT AND 18F-FDG AVIDITY AS COMPARED TO NMZL

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Introduction. Marginal zone lymphomas (MZLs) represent a heterogeneous group of indolent lymphomas arising from memory B lymphocytes within the marginal zone of the secondary lymphoid follicles. MZLs develop in spleen and mucosa-associated lymphoid tissue (MALT), rarely in lymph nodes. The updated WHO classification of lymphoid neoplasms grouped MZLs in three specific subtypes: extranodal MZL (EMZL) or MALT type, splenic MZL (SMZL) and nodal MZL (NMZL). Due to the heterogeneity and rarity of these lymphomas, a risk stratified approach is currently lacking. Our study aims to identify whether integration of several clinical, laboratory and imaging parameters may correlate with the outcome of the different MZL subtypes. *Materials and Methods.* 19 patients (11 females and 8 males), aged between 38 to 84 years, with diagnosis of MZL, were evaluated. Histological subtypes were represented as follows: 12 patients (63.2%) with EMZL [site distribution: stomach (3), lung (3), parotid gland (3), orbit (2) and skin (1)]; 6 NMZL (31,6%) and 1 SMZL (5,2%). At the time of diagnosis, all patients underwent evaluation of basal parameters: clinical and pathology data (sex, age, stage, IPI, histological subtype, Ki67); blood count, LDH, b2-microglobulin and inflammatory markers (ESR, RCP, D-dimer and ferritin); morphological (ultrasound, CT or MRI) and functional (PET/CT) imaging scans (Table 1). PET/CT scans were performed in 15 out of 19 patients; FDG avidity expressed as SUV max was evaluated and included. In case of multiple lesions with different FDG avidity, the higher SUVmax in the patient was considered for statistical analysis. 15 out of 19 patients underwent standard treatments for MZL (steroids, antibiotics, immunotherapies, chemotherapy, radiotherapy or surgery). At the end of treatment, re-evaluation was performed in 12 of the 15 treated patients. The statistical analyses were performed by using SPSS package. *Results*. Due to the presence of only one SMZL patient in the whole group, we decided to exclude this subtype and to compare EMZL (12 patients) *versus* NMZL (6 patients) groups (Table 1). The analysis showed significant differences between the two groups in terms of basal platelets count (147330

vs 259000 cells/mmc, NMZL *vs* EMZL respectively, p=0.018) and basal SUVmax (3.30 *vs* 8.28 NMZL *vs* EMZL respectively, p=0.040). Notably, we found lower ESR values in the NMZL group (6.60 mm/h) compared with the EMZL one (27.00 mm/h) without reaching statistic significance (p=0.100). All the other parameters evaluated did not show significant differences. *Conclusions*. We found a close correlation between EMZL subtype, level of platelet count and SUVmax. These data suggest that increased PET avidity may depend on marked systemic inflammatory stimuli triggered by EMZL rather than NMZL diseases. Further investigations with an enlarged cohort of patients will be needed to confirm these data and to design an integrated score for risk stratification of MZLs.

Table 1. Characteristic of patients and frequency.

	EMZL 12 pts	NMZL 6 pts	SMZL 1 pts
Sex	2 M/4 F	6 M/6 F	1F
Age of diagnosis	38-82 y.o.	46-73 y.o.	84 y.o.
Site	Stomach (3 pts) Lung (3 pts) Parotid gland (3 pts) Orbit (2 pts) Skin (1 pts)	N. A.	N.A.
Platelets basal average	259,0 x10^3 cells/mmc (sd 78,40)	147,3 x10^3 cells/mmc (sd 98,09)	N.A.
ESR basal average	27,2 mm/h (sd 25,48)	6,6 mm/h (sd 2,51)	N.A.
SUV max average	8,28 (sd 3,53)	3,3 (sd 1,40)	N.A.

Abbreviation: EMZL= Extranodal Marginal Zone Lymphomas. NMZL= Nodal Marginal Zone Lymphomas. SMZL= Splenic Marginal Zone Lymphomas. n pts=number of patients. M=male. F=female. Y.o.=years old. N.A.=not applicable. sd=standard deviation. ESR=Erythrocyte Sedimentation Rate. SUV=Standardized uptake value.

PO-100

FIRST LINE TREATMENTI FOR HIGH-RISK DLBCL IN THE RITUXIMAB ERA: HIGH-DOSE CHEMOTHERAPY WITH AUTOLOGOUS STEM CELL TRANSPLANTATION, AN INTENTION TO TREAT-ANALYSYS

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Introduction. R-CHOP is considered to be the standard treatment for patients (pts) with newly diagnosed DLBCL. Treatment results are still unsatisfactory, particularly in high IPI score patients. We analyzed safety and effectiveness of R-CHOP followed by salvage chemotherapy and ASCT for patients with young (<65 years) high-risk DLBCL, defined by an age-adjusted IPI score of 2/3, for whom from 2004 on our institutional guidelines recommended ASCT as consolidation. We analyzed prognostic factors in this group. *Methods.* The treatment program consisted of 4 cycles R-CHOP-14 followed by 3 cycles of a DHAP-like salvage regimen, R-MICMA (Sorà *et al.,* Cancer 2006), and consolidation with Busulfan-Melphalan supported with ASCT. We observed 76 consecutive patients (median age 50 years, range 15-64 years; 32 females and 44 males) diagnosed between May 2004 and January 2013 with DLBCL who had an age-adjusted IPI score of 2 or 3. Response was assessed according to Cheson criteria (Cheson *et al.,* JCO 1999). *Results.* Nine of 76 patients (12%) were not eligible for the treatment program that included ASCT. Reasons were important comorbidities in 6 pts (1 cardiac, 2 neurologic, 1 hepatic, 1 hematologic, 1 renal) and start of another treatment regimen (CODOX-M/IVAC in the suspicion of a Burkitt lymphoma) in 3 pts. Response after 4 cycles R-CHOP was CR/CRu in 40/67 pts (60%), PR in 21/67 pts (31%) and NR in 6/67 (9%). Sixty-one patients went on to salvage chemotherapy with R-MICMA, while 6 pts in CR/CRu continued R-CHOP, and 53 pts were transplanted. Reasons not to proceed to transplant were progressive disease (3 pts), infections (3 pts), mobilization failure (1 pt) and patient's decision (1 pt). The 3-year EFS and OS of the entire group of 76 patients were 67% (95% CI, 55-76) and 71% (95% CI, 59-80%), respectively. The 3-year EFS and OS of transplanted patients were 70% (95% CI, 55-80) and 76% (95% CI, 62-85). Factors associated with inferior EFS were age-adjusted IPI score (2 *vs.* 3, p=0.004) and disease status after 4 cycles R-CHOP (p=0.01) in univariate and multivariate analysis. These differences were also retained in the group of patients who received ASCT, with a three-years EFS of 78% in pts with an age-adjusted IPI score 2 *vs* 46 % in pts with an age-adjusted IPI score 3 (p=0.003), suggesting that ASCT is insufficient for highest risk patients. *Conclusions.* Our findings of an intention-to-treat, single centre experience indicate that 88% of patients with high-risk DLBCL and age <65 years are eligible for a treatment strategy that includes ASCT, and 70% will eventually receive ASCT as part of their first-line treatment. Consolidation with upfront ASCT for high-risk DLBCL is a feasible and promising therapy also in the Rituximab era, but there are still subsets of patients that continue to have a poor prognosis despite ASCT, and addition of new biologic drugs, as tyrosine kinase inhibitors, have to be tested to improve outcome in these patients.

PO-101

PROPHYLAXIS WITH LAMIVUDINE IN MAINTENANCE TREATMENT WITH RITUXIMAB IN NON HODGKIN LYMPHOMA CD20+

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In literature, sporadic Hepatitis B Virus (HBV) reactivation cases are reported not only in patients treated with Rituximab-based regimens but also in patient after maintenance with Rituximab single treatment. The aim of this work is to evaluate how many HBV reactivation occurred among patients with positivity of Hepatitis B core antigen (HBcAB +) and Hepatitis B surface antigen negative (HBsAg-) who underwent to maintenance treatment with Rituximab as single agent. We analyzed retrospectively the prevalence of HBV reactivation among patients with diagnosis of Non Hodgkin Lymphoma CD20+ HBcAb +/HBsAg– who underwent to maintenance treatment with Rituximab after standard Rituximab-based chemoterapy schedule. From January 2007 to December 2013, in our Unit, 92 patients with diagnosis of Non Hodgkin Lymphoma CD20+ underwent, after standard chemoterapy schedule Rituximab-based , to maintenance treatment with Rituximab as single agent (schedule: 375 mg/mq every 3 months for 2 years). Patients were previously treated with different chemotherapy treatments: 42% (39/92) with R-CHOP; 50% (46/92) with R-FN; 3% (3/92) with R-F; 4% (4/92) with R-Chlorambucil. None of these patients received prophylactic treatment with lamivudine during induction or maintenance. All the patients were given blood tests for HBV (HBsAg; HBsAb; HBeAg; HBeAb; HBcAb) before starting maintenance treatment and liver function tests before each administration of Rituximab.

19% of the patients (18/92) were HBcAb positive. 65% of the patients (60/92) completed the maintenance treatment and 28% of them were HBcAb positive (7/25): only in one of these patients occurred the HBV reactivation (median follow up: 24 months). 28% of the patients (26/92) are still in therapy with Rituximab and 11% of them are HBcAb positive (3/26): all these patients are at risk for HBV reactivation too. In patients HBcAb +/ HBsAg– in maintenance with Rituximab as single agent, is suggested prophylaxis with lamivudine. In our retrospective study, HBcAb +/ HBsAg- patients didn't receive any prophylactic treatment with lamivudine during the maintenance treatment with Rituximab and the HBV reactivation occurred only in one patient HBcAb+/HBsAg-, three months after the end of the maintenance therapy. More ambitious prospective studies are required to establish the clinical utility of prophylactic treatment with lamivudine during Rituximab-based-maintenance.

PO-102

PRIMARY HEPATIC NON-HODGKIN'S LYMPHOMA AND CORRELATION WITH HCV INFECTION: EXCELLENT RESULTS WITH CONVENTIONAL CHEMOTHERAPY

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Primary Hepatic (PHL) non-Hodgkin's Lymphoma is a rare entity, frequently associated with a poor prognosis. PHL was first described in 1965 by Ata el al and in 1986 Caccamo *et al.* defined PHL as a localized

lymphoma, limited to the liver without extrahepatic involvement. Small series of PHL have been reported, suggesting a non-fortuitous association with Hepatitis C Virus (HCV) infection. The prognosis is believed to be dismal, with early recurrence and short survival. To date, less than 150 cases have been published. Eleven adult consecutive patients observed in our Division from 1990 to 2013 (median age: 58 years) fulfilled the diagnostic criteria for Primary Hepatic Lymphoma. Our series of patients were derived from 1083 patients with non-Hodgkin's Lymphoma observed in our institution in the same period (*i.e.* with a prevalence of 1.0% for PHL). We performed a study of the viral status and the result of cytotoxic treatment. The disease occurred in middle-aged patients (median age: 58 years). The main presenting complaint was right upper quadrant abdominal pain (4/11 patients). Tumor markers (Alfa-fetoprotein and CEA) were normal in 8 patients tested. Liver scans demonstrated either a solitary nodule or multiple lesions. Pathologic examination revealed diffuse large B cell lymphoma in seven patients, two cases of follicular lymphoma, one of small lymphocytic lymphoma and one case of T cell lymphoma. Eight patients (72%) were HCV-positive. Eight patients were treated with CHOP regimen (6 CHOP and 2 R-CHOP), two patients with R-FN, while a patient with a single focal lesion underwent to surgical treatment. The complete remission rate was 100% (11/11) after frontline therapy, and only one patient relapsed but underwent remission after additional chemotherapy courses; one of these patients, who had HCV-related cirrhosis, died because of hepato-renal syndrome, and another one died because of Acute Myeloid Leukemia. Our study confirms the rarity of PHL. In our Division, the outcome of patients with PHL, who are treated with combination chemotherapy, seems excellent. The frequent association of PHL with HCV infection suggests a possible role of this virus in lymphomagenesis. HCV-infection does not appear to influence the outcome.

Monoclonal Gammopathies and Multiple Myeloma

PO-103

PERIPHERAL BLOOD ABSOLUTE MONOCYTE COUNT AS PREDICTOR OF OVERALL SURVIVAL IN MULTIPLE MYELOMA

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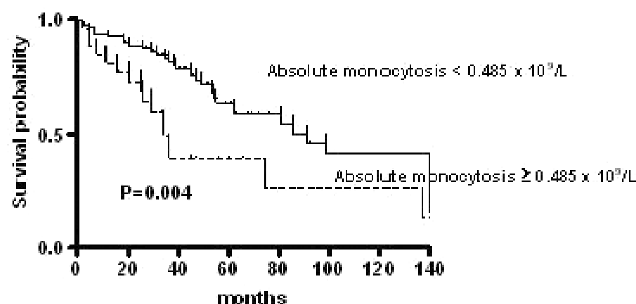
Introduction. The tumor microenvironment, including tumor-infiltrating lymphocytes and myeloid-derived cells, is an important factor in the pathogenesis and clinical behavior of different neoplasms. However, the prognostic significance of peripheral monocytes in multiple myeloma (MM) was never assessed thus far. *Methods.* In a retrospective unselected cohort including 120 patients diagnosed as having active MM at our institution over a 10-year period (2000-2010) we evaluated the prognostic impact of absolute monocyte count (AMC), absolute lymphocyte count (ALC) and lymphocyte/monocyte ratio (LMR). *Results.* Median age of patients was 69 years (range, 41-86 years) and there were 64 males and 56 females. Patient distribution according to ISS was as follows: ISS 1, 75 cases; ISS 2-3, 45 cases. After a median follow-up time of 39 months (range, 2-160 months) 53 patients died while median OS was 52 months. Looking at the prognostic impact of ALC,AMC and LMR different thresholds were set. While ALC or LMR failed to demonstrate any impact on clinical outcome of MM patients in terms of overall survival (OS), a cut-off value of 485 x mmc for AMC, corresponding to 75th percentile, was strongly associated with OS. Survival curve according to AMC is presented below. As shown, median survival of patients with AMC<485 x mmc at the time of diagnosis was 91 months therefore significantly longer than median survival of patients with AMC ≥485 x mmc (34 months) [HR, 2.67 (95% CI, 2.12-3.22); P=0.004]. No correlation could be found between AMC and ISS score (P=0.719), response to therapy (P=0.966) and comorbidity index as assessed by ACE-27 score (P=0.135). In contrast, an inverse correlation between patient age and AMC characterized our cohort (P=0.04) (Table 1). *Conclusions.* In summary, AMC at diagnosis, as a simple index which reflects tumor microenvironment, predicts clinical outcome in MM patients. AMC is an objective, reproducible and cost effective test, which can easily be obtained from standard blood count. Additional studies on AMC are warranted in MM patients to better understand the roles of monocytes in this disease.

Table 1.



PO-104

A NOVEL IMMUNOPHENOTYPIC PROFILE FOR TUMOR PLASMA CELLS DISCRIMINATION

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Introduction. Multiple myeloma (MM) is a neoplastic disorder characterized by clonal proliferation of malignant plasma cells (PCs) in bone marrow (BM). MM is still an incurable disease and novel therapeutic drugs such as monoclonal antibody (mAb) could improve MM treatment. Flow cytometry is considered an indispensable tool to con-

firm diagnosis, as it allows to discriminate between normal and monoclonal plasma cells. The expression of a wide panel of surface markers provided by the "Ninth Human Leukocyte Differentiation Antigens" workshop (HLDA9) was investigated in MM patients. The aim of this study was to investigate the expression of new antigens in MM PCs in order to expand knowledge on normal and tumor PCs immunophenotype, to investigate new potential prognostic factors and targets for therapy. *Methods.* The expression of 82 surface markers provided by the HLDA9 were analyzed by flow cytometry in 20 MM patients. PCs were identified by CD38 and CD138 coexpression. The antigens positive in at least 60% of the samples (CD150, CD48, CD229, CD352, CD319, CD272, CD86, CD200 and CD184) were subsequently tested by FACS CANTO II flow cytometer equipped with FACS Diva software in 38 patients: 24 newly diagnosed MM (Dia), 8 relapsed MM (Rel), 6 plasma cell leukemia (PCL), and 13 subjects without hematological diseases (Healthy). The percentage of positive samples, containing >20% positive PCs, has been evaluated together with the mean fluorescence intensity (MFI) of antigen expression. *Results.* Surface molecules provided by the HLDA9 expressed in almost 60% of MM samples were evaluated in 38 samples from MG and 13 Healthy subjects. Results are shown in Table 1. Positive samples for CD150 were more represented in Healthy than in MM Dia and MM Rel (p=0.015 and p=0.002, respectively); CD86+ samples were more represented in Healthy than in MM Dia (p=0.006); CD200+ samples were more frequent in MM Dia than in Healthy (p=0.001) and MM Rel (p=0.001). From the analysis of MFI CD150, CD319 and CD352 showed higher values in Healthy than in MM Dia (p=0.012, p=<0.001, p<0.001, respectively); MFI of CD352 was also higher in Healthy than MM Rel (p=0.008), while MFI of CD229, CD319 was more intense in Healthy than in PCL (p=0.045, p=0.004, respectively). *Conclusions.* Our results indicate the possibility to identify PCs by their surface expression of CD272, CD319, CD229 and CD48 that could be added to routinely tested markers by flow cytometry. These antigens, if our data will be confirmed on a larger number of samples, could be considered targets for therapy. From these preliminary data we can suggest that CD150, CD86 and CD200 are useful markers to discriminate between tumor and normal PCs. Moreover, the intensity of expression of CD150, CD319 and CD352 could help in the differentiation between Healthy and MM PCs. Further studies on a large clinical controlled trial is needed to provide evidence of the prognostic relevance of these markers.

Table 1. Percentages of positive samples in Healthy, newly diagnosed MM, relapsed MM and PCL subjects.

	Healthy	MM Dia	MM Rel	PCL
CD150	100	62.5	25	66.7
CD86	100	58.3	87.5	83.3
CD319	100	100	100	100
CD229	100	100	100	100
CD184	76.9	70.8	37.5	33.3
CD272	100	95.8	87.5	83.3
CD200	23	87.5	12.5	50
CD352	100	79.2	62.5	66.7
CD48	100	100	87.5	83.3

PO-105

IN MGUS GRANULOCYTES ARE DYSFUNCTIONAL AND IMMUNOSUPPRESSIVE

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Introduction. Our previous work showed that in Multiple Myeloma (MM) the immune function is impaired, including immunosuppressive properties of myeloid compartment. It is currently unknown if immune dysfunction occurs in MGUS as well. *Aim*. Providing a detailed analysis of granulocyte function at the steady-state in MGUS. *Methods*. Using oligonucleotide microarrays we first evaluated the gene expression profile of granulocytes at the steady state in 3 MGUS and 3 healthy subjects matched for sex and age. Then, we validated preliminary findings in granulocytes and serum from peripheral blood in 60 consecutive newly diagnosed MGUS and 15 healthy subjects, measuring: i) surface markers of activation detectable by flow cytometry: CD11b, CD14, CD16; ii) Arg-1 and BV8 in RT-PCR. In addition, we tested the immunosuppressive properties of granulocytes isolated from MGUS cultured *in vitro* with T-lymphocytes obtained from healthy subjects. *Results*. We found 708 genes differentially expressed (467 up- and 241 down regulated) in MGUS *versus* healthy granulocytes at the steady state. The set of annotated, differentially expressed genes could be functionally organized by "gene ontology" (http://www. geneontology.org/) into the following major categories: i) receptors and signal transduction (including up-regulation of CD14, Toll-like receptor 5 (TLR-5), IL-7 Receptor (CD127), IL-11 receptor, TGF-beta receptor 2, hematopoietic cells kinase (HCK), IFNAR1); ii) negative regulation of adaptive immune response (including up regulation of CD127, STAT6, IFNAR1, BV8 and down regulation of p50, p65, NFKBIA, IL8, ELK-1, HIF-1 alpha, CEBP-beta, CEBP-zeta). These data suggested a reduced activation status and impaired function that we validated with several techniques (flow cytometry, RT-PCR, ELISA) as follows: i) Arginase (Arg-1) expression was six times higher in MGUS than healthy subjects (p=.03), as soluble form in serum (60 *versus* 30 ng/dL, p=.03); ii) BV8 expression was two times higher in MGUS than healthy subjects (p=.02); iii) Activation markers CD16 and CD11b were reduced (respectively p=.001, p=.02). Finally, in presence of MGUS granulocytes, PHA-P stimulated T-lymphocytes isolated from healthy donors missed the expression of activation markers such CD71, CD69, CD25, CD3Œ∂ in MGUS. *Conclusions*. Granulocytic impairment is present in MGUS patients due to increased expression of genes that negatively regulate adaptive immune response. Further studies will identify those could contribute to immunological paresis in the progression from MGUS to MM.

PO-106

FEASIBILITY OF A SECOND PERIPHERAL BLOOD STEM CELL MOBILIZATION AFTER A PREVIOUS TRANSPLANT PROCEDURE IN RELAPSED MULTIPLE MYELOMA

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Background. Autologous stem cell transplant (ASCT) does still represent the standard of care for young multiple myeloma (MM) patients. Re-transplantation can be taken into consideration for patients esperiencing a late relapse (>2 years after a previous transplant) who have a sufficient amount of stored stem cells. Little is known, at present, on the feasibility of a further peripheral blood stem cell (PBSC) mobilization procedure in patients who had received a single or a double autologous stem cell transplant, as high-dose melphalan or busulfan are toxic to stem cells. *Methods*. In the present study we retrospectively analyzed the outcome of 23 patients (14M, 9F, median age=62.5yrs) with MM relapsing after a single (n=4) or a double (n=19) autologous stem cell transplant who were submitted to a second mobilization procedure at 4 Italian Centres. All the patients had received high-dose melphalan as preparative regimen for all the transplants; Busulfan was added in two cases who had been treated with a double transplant. Fifteen patients had received mantainance therapy with either alpha interferon (N=8) or high-dose dexamethasone. Reinduction therapy upon relapse was Bortezomib-based in 21 cases; a very-good partial response (VGPR) or better was obtained in 9 patients. Median time from last transplant and second stem cell collection was 75 months (range 44-209 months). *Results*. PBSC mobilization was performed using Cyclophosphamide 4g/sqm+G-CSF in 13 cases and G-CSF alone in 10 patients; Plerixafor was added in 14 cases. One out of 23 patients failed to mobilize an adequate amount of CD34+ cells/kg to support the transplant procedure. In the remaining cases PBSC collection was successful in a median of 2 apheresys. The post-transplant PBSC collection, however,

was significantly poorer as compared to the prevoius one in all the cases (3.36 x 106CD34+ cells/kg *vs* 8.3 x 106 CD34+ cells/kg, p<0.001). PBSC yield was not influenced by number of transplants, response to therapy and treatment with Interferon. Twenty-two patients were submitted to ASCT; hematopoietic recovery was 11 days for PMN > 500/mmc and 13 days for platelets > 20000/mmc. Grade III-IV non hematological toxicity of the whole transplant program was observed in 3/22 (13%) cases. Median progression-free survival was 27 months. *Conclusions*. Although these data should be confirmed in a larger prospective study, a second PBSC mobilization followed by ASCT seems to be is feasible in patients with MM who have previously received a transplant procedure.

PO-107

DISTINCT GENE AND MIRNA EXPRESSION PROFILES IN SEQUENTIAL MYELOMA SAMPLES AT DIAGNOSIS AND RELAPSE

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Introduction. Multiple myeloma (MM) is characterized by a highly heterogeneous clinical course, which may be only partially explained by the presence of major genetic alterations. High-resolution technologies are improving the knowledge of the molecular mechanisms underlying MM disease evolution. To provide useful information concerning the molecular alterations associated with tumor progression in MM, we generated global gene and miRNA expression profiles in sequential samples of MM patients, evaluated at diagnosis and relapse. *Methods.* Global gene expression profiles of highly purified plasma cells (PCs) from 17 symptomatic MM and 2 primary PC leukemia (PCL) patients were generated at diagnosis and relapse after first line therapy, by means of Affymetrix Gene 1.0 ST array. Twelve (11 MM, 1 PCL) paired samples for which material was available were also profiled on Affymetrix miRNA 3.0 array. Unsupervised and paired supervised analyses were carried out by dChip and SAM software, respectively. Functional annotation studies were performed using DAVID 6.7 and miRBase tools. Main genomic aberrations were investigated by FISH analysis. *Results.* Concerning major genomic aberrations, acquisition of del(13) and del(17) were respectively observed in one and two relapsed MMs of 15 analyzed cases; 1q gain aberration was acquired in 2 MMs and 1 PCL, among 11 tested patients. In particular, 1q gain and del(17) were both acquired in one relapsed MM. Main IgH chromosomal translocations found at onset were confirmed at relapse. No IgH translocation was acquired in any patient at relapse. Paired supervised analysis identified 171 differentially expressed genes, all but one up-regulated in baseline MM/PCL compared to relapsed samples. In particular, a stronger overexpression pattern at diagnosis was observed in seven patients: the two PCL cases carrying the t(11;14) and t(14;16) respectively, the two MAF-translocated MMs and 3 MMs without major IgH translocations, two of which acquired del(17) at relapse. Deregulated transcripts were mainly specific of genes involved in RNA processing, including spliceosome components, and comprising also proteasome genes. Furthermore, we evidenced the differential expression of ATG5 and M6PR, implicated in drug response mechanisms involving autophagy process, and CRBN, whose downregulation has been linked to drug resistance in MM. Furthermore, five miRNAs were found positively modulated in relapse compared to diagnosis phase; among them, miR-25, a negative regulator of TP53, was found to be associated with high-risk MM and miR-4428 is a novel candidate miRNA in human B cell malignancies. *Conclusions.* Microarray technologies may be useful in identifying genes and miRNAs specifically deregulated in MM progression. These data may contribute to our understanding of mechanisms involved in drug resistance and clonal evolution in MM, and to improve the risk-stratification of patients.

PO-108

NOVEL THERAPEUTIC TARGETS IN MULTIPLE MYELOMA: PROTEIN KINASE CSNK1A DEPENDENT SIGNALING NETWORK

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Introduction. Multiple myeloma (MM) is the second most frequent hematologic malignancy, accounting for about 10% of all blood tumors. Dwelling of MM in the bone marrow microenvironment favours uncontrolled growth, genetic clonal selection and inherent drug resistance. MM cells and stromal cells also produce osteoclast-promoting cytokines as well as osteoblast inhibitory factors, such as potent antagonist of the Wnt pathway, which, in turn, is essential for osteoblastogenesis. Clinically, MM patients face the repeated relapses as well as the devastating bone disease. Novel therapeutic targets are thus urgently needed. Casein Kinase 1α (CK1α) is a serine/threonine kinase essential for the function of signaling pathways that are involved in MM pathobiology: the canonical Wingless (Wnt)/β-catenin cascade; the Hedgehog (Hh) pathway; the NF-κB pathway; p53-driven response, here by stimulating the binding to MDM2 and p53 inhibition. However, despite these notions could suggest a role of CK1α in growth, survival and proliferation of malignant plasma cells, and tumor-microenvironment interactions/osteoblastogeneis, its function in MM and related bone disease has, to the best of our knowledge, never been investigated. *Methods.* CK1α expression and activity has been analyzed in MM cells and controls; the consequences on MM cell survival and proliferation and osteoblastogenesis of CK1α inhibition with small ATP-competitive compounds or by RNA interference has been investigated. CK1α-dependent signaling events have been analyzed by different methods. *Results.* We analyzed a number of data sets in available repositories and found that CK1α is significantly over expressed across the evolution from normal to highly malignant plasma cells in most of the human datasets as well as in a mouse myeloma model. CSNK1α mRNA and CK1α protein were found highly expressed expression in purified CD138+ malignant plasma cells from MM patients. Treating MM cells with the CK1α inhibitor D4476 or RNA interference caused a variable amount of apoptosis and growth arrest, even in co-cultures with protective BM stromal cells, which were instread spared by the cytotoxicity. Surprisingly, the combined treatment with bortezomib caused a much stronger cytotoxic effect on MM cells than the single treatments. Analysis of signaling networks revealed a strong inhibition of β-catenin phosphorylation and Akt activation as well profound perturbations of the ER stress-unfolded protein response pathways, especially of the IRE1α and PERK-dependent branches. *Conclusions.* The data we have collected so far allow concluding that CK1α could be a growth-propelling kinase in MM. The identification of the exact mechanism of action and of the pathways targeted by CK1α in MM plasma cells is the subject of future research.

PO-109

NOTCH TARGETING PREVENTS MULTIPLE MYELOMA ASSOCIATED OSTEOCLASTOGENESIS

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Introduction. Multiple myeloma is an incurable hematological tumor stemming from malignant plasma cells. MM cells accumulate in the bone marrow (BM) and establish complex interactions with normal BM stroma, which promotes tumor survival, bone disease and drug resistance. Recent evidence indicate that Notch pathway is deregulated in MM and plays a role in the pathogenesis of this tumor by modulating tumor cell biology and the crosstalk between MM cells and the BM niche. Activation of the Notch signaling is mainly due to the the aberrant expression of two of the Notch ligands, Jag1 and Jag2, that causes an increase in the ability of MM cells to trigger Notch activation in neighboring tumor cells and cells of the BM niche. In this work, we

investigated the contribution of Jag1/2 to the osteoclastogenic potential of MM. *Methods*. The γ-secretase inhibitor DAPT was used at a final concentration of 50μM. Osteoclasts (OCL) differentiation of Raw264.7 cells was induced by treatment with 50ng/ml mRANKL or co-culturing with MM cells or their conditioned medium. After 5-7days cells were stained using the TRAP Kit and counted. For bone resorption assay, Raw264.7 cells were cultured on Osteo Assay Surface plates under differentiation conditions for 7-10 days. Images of the resorbed areas were captured and the % of resorbed area was measured using the Wimasis image analysis software. Select RNAiTM siRNA system (Invitrogen) was used according to the manufacturer instructions for the selective inhibition of Jag1 and Jag2. Transfection was performed by electroporation using two plasmids coding the intracellular Notch1 and Notch2.Total RNA was isolated using TRI-Reagent. cDNA was prepared through MMLV-RT, then quantitative PCR was performed by Maxima SYBR Green qPCR Master Mix. ELISA Assay was performed using biotin-conjugated goat anti-human RANKL (Merck-Millipore) and Streptavidin-HRP-labeled secondary antibody. Flow citometry was performed using an anti-human RANKL antibody (Abcam) and a Alexa488-conjugated secondary antibody (Life Technologies). Jag1 recombinant peptide was used at 0.5μg/ml. anti-RANKL neutralizing antibody was used at 0.1μg/ml. *Results*. Our results indicate that Notch2 signaling is essential for OCL differentiation and activity. Furthermore, this work supports the hypothesis that Jag ligands expressed by MM cells promote OCLs differentiation in two different ways: through the release of RANKL or by direct contact with OCL precursors. Jag ligands are able to mediate the interactions of MM cells with the BM niche which further promotes MM cell osteoclastogenic ability. Indeed, Jag1/2 silencing inhibits MM cell ability to interact with BM stromal cells and to induce osteoclastogenesis. *Conclusions*. Our work demonstrates that Notch signaling drives MM-induced osteoclastogenesis and osteolysis and suggest that Jag1/2 might present new promising therapeutic targets to reduce MM-associated bone disease.

PO-110

RELATIONSHIP BETWEEN CIRCULATING PLASMA CELLS AND CYTOGENETIC RISK IN PATIENTS WITH MULTIPLE MYELOMA

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Multiple Myeloma (MM) is a clonal B-cell disorder characterized by accumulation of malignant plasma cells (PC) in the bone marrow (BM). Circulating plasma cells (PCs) can be detected in the peripheral blood of a significant proportion of patients with MM and their presence is a well-known prognostic factor. Indeed, the appearance of circulating PCs in the blood could indicate relative indipendence from adhesion to the microenvironment and, therefore, signifies more aggressive disease. In this study, we examined the relationship between the number of PCs and citogenetic risk in patients with newly diagnosed MM. We analyzed peripheral blood from patients with Monoclonal Gammopathy of Undetermined Significance (MGUS; n=15), Smoldering Myeloma (SM; n=28), Solitary Plasmacytomas (SP; n=3) and active MM (n=105). These patients were followed by the UOC Ematologia at the "Mazzoni" Hospital from January 2006 to December 2013, with a median follow-up of 25 months. We analyzed clinical, laboratory and cytogenetic data of patients with active MM. However, cytogenetic analysis was not evaluable for 15 patients. The number of PCs was detected by flow cytometry using a simple two-colour approach. Cells were stained with fluorescence-labeled CD38 and CD45 antibodies and 50,000 events were acquired and analyzed for each patient. PCs were identified by gating on CD38bright+/CD45- cells. Using a receiver operating characteristics (ROC) analysis, we assessed that ≥41circulating PCs is the optimal cut-off for defining poor prognosis. The 8-years probability of Overall Survival (OS) and Progression-Free Survival (PFS) in patients with <41 and ≥41circulating PCs, was 32% *vs* 8% (p=0.017) and 29% *vs* 0% (p=0.0008), respectively. Patients with high-risk cytogenetics (n=24) had poor prognosis, independently of circulating PCs (PC<41 *vs* PC≥41: OS=0% *vs* OS=16%, p=n.s.; PFS=0% *vs* 17%, p=n.s.). Patients with standard-risk cytogenetics (n=66) showed a better prognosis associated to a lower number of circulating PCs (PC<41 *vs* PC≥41: OS=36% *vs* 10%, p=0.026; PFS=37% *vs* 0%, p=0.0001). These data were confirmed by multivariate analysis (Cox model) for the subgroup with standard-risk cytogenetics, in which the presence of ≥41 circulating PCs, older age, DS stage >I and lack of maintenance therapy, adversely affected OS and PFS. All patients with SP showed no circulating PCs. In all cases of MGUS or SM, circulating PCs, when detected, were <20. In summary, our results suggest that the quantification of circulating PCs by flow cytometry could provide useful prognostic information for newly diagnosed MM patients with standard-risk cytogenetics.

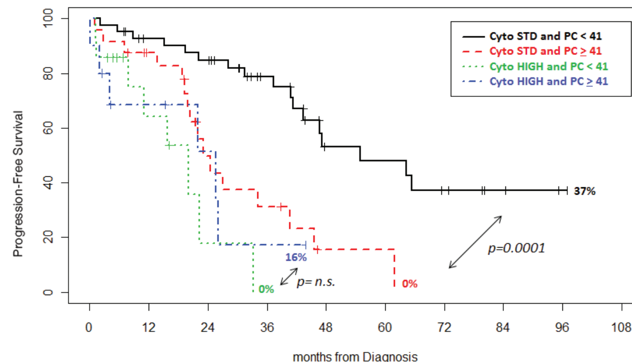

Figure 1.

PO-111

IN VITRO IMPAIRMENT OF EZH2 IN HUMAN MULTIPLE MYELOMA CELL LINES CAUSES A DECREASE IN CELL PROLIFERATION

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Introduction. EZH2 (Enhancer of Zeste 2) is the SET containing catalytic subunit of the Polycomb Repressive Complex 2 (PRC2), which is comprised of SUZ12, EED, RbAp48 and JARID2 among other interacting proteins that function to repress transcription through H3K27 trimethylation. EZH2 is over-expressed in solid tumors and is associated with poor prognosis and metastatic progression. EZH2 knock-down, blocks proliferation, cell invasion, tumor growth and metastasis, whereas its over-expression produces an oncogenic phenotype. In prostate cancer, the oncogenic function of EZH2 requires the activity of MMSET (Multiple myeloma Set Domain) protein (Asangani IA. *et al.*, Molecular Cell 2013). Our previous studies indicated that EZH2 is up-regulated in tumor progression in multiple myeloma (MM). To investigate the role of EZH2 in MM, we performed knock-down experiments in human myeloma cell lines (HMCLs). In particular, we focused on two HMCLs characterized by the t(4;14) translocation associated with MMSET over-expression. *Methods*. HEK 293T cells have been transfected with a lentiviral vector pGIPZ EZH2 sh and with an empty pGIPZ vector as control (Thermo Scientific Open Biosystems expression Arrest GIPZ Lentiviral shRNA mir). The supernatants from GFP positive cells were used to infect HMCLs KMS11 and KMS34. GFP positive cells were collected and processed for quantitative real time PCR (Q-RT-PCR) (TaqMan miRNA assays, Applied Biosystems), and immunoblotting (WB). *Results*. To investigate the role of EZH2 in MM, we impaired its expression in KMS11 and KMS34 HMCLs both characterized by the t(4;14) translocation. The impairment of EZH2 expression, confirmed by Q-RT-PCR and WB analyses of GFP positive cells, was associated with a 40% reduction in cell proliferation. The analysis of MMSET protein amount in KMS11 HMCL GFP positive cells showed a 60% reduction if compared with GFP positive control cells. *Conclusions*. Our preliminary data suggest that impairment of EZH2 is able to reduce cell proliferation in *in vitro* myeloma cell lines. In addition, EZH2 knock-down induces a significant reduction of MMSET protein level. This is in agreement with the model described in prostate cancer and are currently under investigation in our laboratory.

PO-112

BONE MARROW-MEDIATED DRUG RESISTANCE IS PROMOTED BY JAGGED-INDUCED NOTCH SIGNALING IN MULTIPLE MYELOMA

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Background. Multiple myeloma (MM) is an hematologic tumor caused by the accumulation of malignant plasma cells in the bone marrow (BM). The dysregulated expression of two Notch ligands, Jagged1 and Jagged2, hyperactivates the Notch pathway both in MM cells and in BM stromal cell (BMSC). Several Notch downstream mediators are involved in MM cell survival and proliferation, *i.e.* IL6, SDF1α, CXCR4, NF-kB, VEGF and IGF. Although treatments with new drugs, such as alkylating agents, proteasome inhibitors and immunomodulatory agents, increased patients'survival, MM remains incurable, principally due to the development of endogenous or BM-mediated drug resistance (DR). Therefore it is crucial to find new therapeutic targets. The aim of this study was to investigate the role of Notch signaling in endogenous and BMSC-promoted DR in MM. *Materials and Methods.* U266 and OPM2 cell lines were maintained in complete RPMI-1640 medium. The BMSC lines NIH3T3 (murine) and HS5 (human) were maintained in complete DMEM medium. MM cells were cultured alone or on a BMSC monolayer for 24h, and subsequently treated with Mitoxantrone, Bortezomib, Melphalan or the vehicle in the presence or the absence of the CXCR4 antagonist AMD3100 for additional 24 hours. BMSCs were previously stained with PKH26 (Sigma-Aldrich) to discriminate them from MM by flow cytometry. Apoptosis was determined by Annexin V-FITC staining. qRT-PCR reactions were carried out in a 7500 Fast Real-time PCR system (Applied Biosystems) using the Maxima™ SYBR Green/ROX qPCR Master Mix (ThermoScientific Inc) using murine or human primer sets to discriminate the source of the RNA molecules. Silencing of Jagged1 and 2 was obtained by transient expression of specific siRNAs. *Results*. The possible role of Notch withdrawal in DR was investigated by silencing Jagged 1 and 2 ligands in MM cell lines OPM-2 and U266. Results showed an increased sensitivity to Bortezomib, Mitoxantrone and Melphalan associated to a decrease in the expression of SDF1α, CXCR4, Bcl-XL, Bcl-2, Survivin and ABCC1. When co-cultured with murine and human BMSCs, MM cells showed increased DR due to: i) increased expression of anti-apoptotic genes in MM cells, *i.e.* Bcl-XL, Bcl-2, Survivin and ABCC1; ii) BMSC release of soluble mediators relevant to MM cells, *i.e.* SDF1α and VEGF. We suggest that these effects may be driven by the reciprocal activation of Notch signaling observed in both cell types and consistently we demonstrated that DR may be significantly reduced by silencing Jagged1 and 2 in MM cells. Finally, the evidence that CXCR4 blockade significantly reduced MM cells resistance to Bortezomib induced by BMCSs, indicates that CXCR4/SDF1α chemokine axis is a key mediator of Notch in MM-associated DR. *Conclusions*. The evidence that Jagged1/2 silencing affects endogenous and BMSC-induced DR in MM cells supports the use of a Jagged-targeted approach in MM therapy alone or in combination with common drugs.

PO-113

ROLE OF BENDAMUSTINE IN RELAPSED AND REFRACTORY MULTIPLE MYELOMA

Cerchione C, Madonna E, Pareto AE, Basile S, Peluso I, Vitagliano O, Cimmino C, Simeone L, Seneca E, Pugliese N, Marano L, Pane F, Catalano L

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Efficacy and tolerance of bendamustine in combination with bortezomib-dexamethasone was evaluated in patients with relapsed and refractory multiple myeloma (rrMM), whose prognosis is severe, so that there is a strong need for new options for the management of these patients. 24 patients, 13 males, 11 females, with rrMM, who had been treated with a schedule Bendamustine-based, were retrospectively analyzed. Median age at diagnosis was 63.2 years (range 39-82) while age at start of treatment was 66 years (range 48-83), and median number of prior lines of treatment was 6.3 (range 4-8). ISS was equally distributed, and cytogenetic characteristics were evaluable in 9 patients, only two of whom had cytogenetic abnormalities, and in particular one of them had del13q and in the other one was observed t(11;14). All the patients had previously been treated with schedule containing bortezomib and lenalidomide, while 90% of them had been treated with melphalan, 77% with cyclophosphamide and 34% with anthracyclines, and 30% had also received radiotherapy. 58% of patients had undergone at least to a single autologous stem cell transplantation. Last treatment before bendamustine was a bortezomib-based regimen in 39%, an IMIDs-based regimen in 49% (a combined bortezomib/IMIDs-based regimen in 27%), while 12% of patients had received other chemotherapies. All patients were relapsed and refractory to last therapies received. Only patients completing at least two two courses of Bendamustine were considered. A total of 87 cycles was administered (median 4.3, range 2-9). In 91% of patients bendamustine was variously associated to bortezomib (66%), or IMIDs (25%) and only in 8% it was combined only with dexametasone. In our schedule, Bendamustine was given, at a median dose of 90 mg/sqm (range total dose: 120-180 mg) on day +1 and +2 every 28 days. After a median follow-up of 6.1 months, median OS from diagnosis was 57.3 months, while median OS from start of Bendamustine was 6.7 months (range 2-19 months). 11/24 patients died for progressive disease. 2/24 patients died for other causes (one for cardiovascular disease and the other one had a gastric cancer). Grade 3 transfusion-dependent anemia occurred in 36% while in 53% grade 3 neutropenia occurred. We observed no severe extrahematologic toxicity, only grade 1 gastrointestinal side effect (nausea), treated by common antiemetic drugs. According to IMWG uniform response criteria, 15 out of 24 evaluable patients achieved a partial response after a median time of 2.4 months with an overall response rate of 62.5%. In particular, for 3 patients of this study. In conclusion, Bendamustine has shown significant efficacy in a particularly severe setting of patients, relapsed and refractory to almost all available therapeutic resources, and in particular cases it could be considered as a bridge to a second autologous or to allogeneic BMT.

Miscellaneous

PO-114

DERANGEMENT OF THE T-CELL REPERTOIRE IN PATIENTS WITH B-CELL NON HODGKIN LYMPHOMA

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Introduction. Although a number of studies suggest that different immune pathways may play a role in the pathogenesis of non Hodgkin lymphomas (NHL), the shape of the T-cell compartment has been only superficially explored in these patients. Focusing on the T-cell receptor (TCR) repertoire pattern, the few available data weakly suggest an overall impairment, however failing to identify specific anomalies potentially mirroring T-cell responses driven by well recognized neoplastic antigens. In our study, we extensively analyzed the TCR repertoire and the distribution of different T-cell subsets -including regulatory T-cells (Treg)- in 30 patients with NHL, by combining flow cytometry and spectratyping. *Methods.* Our study was performed on the peripheral blood of 30 patients and 30 age-matched controls. The TCR repertoire analysis was based on a panel of 24 beta variable (BV) antibodies. Treg were identified by considering the CD4+CD25high+CD127low cell fraction. We then analysed the profile of the third complementarity-determining-region (CDR3) in separated CD4+ and CD8+ cells by spectratyping. After immunomagnetic CD4+/CD8+ cell separation, RNA extraction and reverse transcriptase PCR, CDR3 fragment analysis was performed through capillary electrophoresis. *Results.* While the frequency of CD4+ lymphocyte expansions determined by flow cytometry was similar between patients and controls (1.7% *vs.* 1.7%, p=0.707), NHL patients were characterized by a higher frequency of expanded BVs within the CD8+ subset (5.0% *vs.* 3.2%, p< 0.05). We also showed a higher frequency of CD4+CD25high+CD127low Treg in patients than in controls (mean 2.10% *vs* 1.14%), although this increase was mainly confined to patients with aggressive (mean 2.56%) rather than indolent NHL (mean 1.75%). As regards spectratyping data, the percentage of skewed BVs was significantly higher in patients than in age-matched controls both in CD4+ cells (mean 45% *vs.* 26%, p< 0.0005) and CD8+ cells (mean 88% *vs.* 76%, p< 0.0005). A kinetic evaluation during chemo-immunotherapy clearly highlighted as the CDR3 derangement observed at diagnosis persisted up to 12 months, especially within the CD4+ subset. *Conclusions.* On the whole our data suggest that in patients with B-cell NHL the T-cell immune system is profoundly deranged, as witnessed by the peripheral expansion of both oligoclonal clusters of CD8+ T-cells and Treg as well as by the increased degree of skewing observed within the CDR3 region. All these findings could offer some practical implications. In fact, this condition of immune impairment should be considered when delineating strategies of anti-infective prophylaxis in this clinical setting. Moreover, this functional derangement of the T-cell compartment which is potentially extended to anti-tumor immune responses, should be taken into account while designing new therapeutic strategies which could ideally rely also on the restoration of an effective anti-neoplastic T-cell immunity.

PO-115

ISOLATION, IDENTIFICATION AND PHENOTYPIC CHARACTERIZATION OF EXTRACELLULAR VESICLES IN DIFFERENT HUMAN HEMATOLOGICAL MALIGNANCIES

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Introduction. Extracellular vesicles (EVs), including exosomes, shedding vesicles (microvesicles: MVs) and apoptotic bodies, are membrane vesicles of 40-1,000 nm that are released from many cell types, including red blood cells, platelets, lymphocytes, dendritic cells, endothelial cells and tumour cells. They have a key role not only in the regulation of normal physiological processes, but also in the pathology underlying several diseases. Recently, it was discovered that EVs circulate in bodily fluids of cancer patients and could serve as potential diagnostic biomarkers. However, the presence and role of peripheral circulating EVs in hematological malignancies (HMs) remain unclear. The aim of this study was to investigate the presence of EVs in peripheral blood of HM patients, defining the EVs count, size and phenotype. *Methods.* Peripheral blood samples were obtained prior to treatment from 6 CLL, 16 NHL (follicular, diffuse large B-cell, Waldenstrom), 6 HL, 6 MM, 5 AML, 19 MPN (ET, PV, PMF, CML), 5 MDS and 24 healthy controls. EVs were isolated from serum of peripheral blood by ultracentrifugation steps. For calculation of counts, TruCOUNT beads were added immediately prior to analysis by flow cytometry. For size assessment, Megamix beads with specific size (0.3,0.9, 3.0 micrometers) were used. For phenotype characterization a panel of specific antibodies (anti CD59, CD61, CD62E, CD13, CD33, CD19, CD20, CD38, CD138, CD30, CD15) were used in a FACS analysis. *Results*. Peripheral blood MVs levels were significantly elevated in NHL, HL, CML (p< 0.0001), CLL (p< 0.03), MM and AML (p< 0.01) compared to healthy controls (Figure 1). In some HMs, such as CLL and MDS, the MVs count correlated with stage and risk of malignancy, respectively. All HMs generated distinct population of MVs with different size distribution between 0,3-1micrometers when compared with healthy controls. The peripheral blood MVs expressed a common set of membrane proteins (CD59, CD61 and CD62E) and origin-specific subsets of proteins that likely correlated to malignancy, such as CD19 on CLL and NHL MVs; CD38-CD138 on MM EVs; CD13 on AML, MPN or MDS MVs. *Conclusions*. Patients with different HMs express elevated peripheral blood MVs levels with different size distribution and selectively enriched markers of malignancy. Molecular characterization of HM MVs is in progress.

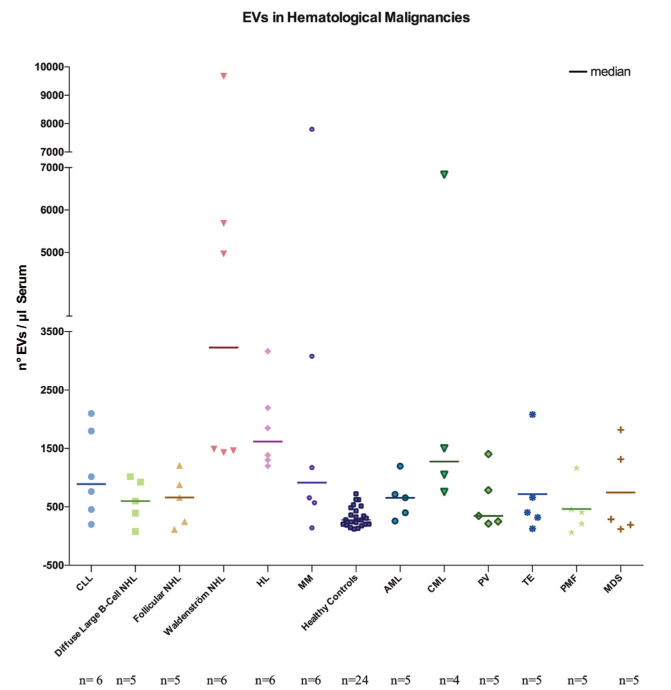

Figure 1.

PO-116

GENOMIC ORGANIZATION AND EVOLUTION OF DOUBLE MINUTES/HOMOGENEOUSLY STAINING REGIONS (DMIN/HSR) WITH MYC AMPLIFICATION IN SOLID TUMORS AND HEMATOLOGICAL MALIGNANCIES

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Introduction. The mechanism for generating double minutes chromosomes (dmin) and homogeneously staining regions (hsr) in cancer is still poorly understood. Local chromosome shattering involving a single-step catastrophic event, termed chromothripsis, has been recently proposed for the generation of clustered chromosomal rearrangements and genomic amplifications in cancers. *Methods.* We used a comprehensive genome-wide analysis combining next-generation sequencing (NGS), SNP array, PCR and FISH, to characterize the MYC amplicons originating from one to four chromosomes.in dmin/hsr structures in seven tumor cell lines. [one acute myeloid leukemia (HL-60), four small cell lung cancer (GLC1DM, GLC1HSR, GLC2, GLC3), and two colon carcinoma (COLO320DM and COLO320HSR)]. Moreover, the fine mapping of hsr insertion sites was accomplished by combining FISH and NGS. Once we identified the hsr insertion sites at nucleotide level, we investigated, by appropriate bioinformatic approaches, the surrounding regions searching for enrichment in segmental duplications, repeats, and motifs potentially associated with genomic instability. Finally, we evaluated the expression level of genes amplified on dmin and interrupted by hsr integration by quantitative RT-PCR. Our investigations were particularly focused on the identification of fusion genes that originated from novel junctions on dmin/hsr. *Results.* By precisely mapping structural variations, we observed the coexistence of heterogeneous amplicons differing in complexity and chromosome content in cell lines derived from the same patient (GLC1DM/HSR and COLO320DM/HSR). Globally, we detected an unprecedented number of hsr insertion sites and determined that amplicon seeding is not driven by any recurrent sequence motif. Finally, we identified two novel fusion genes generated by structural rearrangements within amplicons (5'PVT1/3'EYA1 and 5'PVT1/3'AKT3), resulting in the production of putative shorter EYA1 and AKT3 proteins, respectively. *Conclusions.* Our integrated approach demonstrates that cytogenetics and molecular techniques can complement whole-genome NGS, particularly in complex genomic regions, allowing the detailed resolution of chromosomal aberrations in cancer. Moreover, amplicon heterogeneity observed in our cell lines suggests to exclude chromothripsis as the driving force underlying amplicon genesis in these samples, and invokes a multi-step evolutionary process starting from single-chromosome ancestral episomes. Furthermore, we identified PVT1 as a target of fusion events, confirming its role as a breakpoint hotspot in MYC amplification.

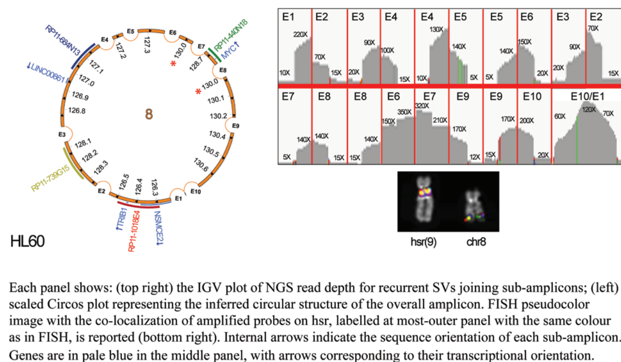

Figure 1. Circular structure of the tandemly repeated chromosome 8 amplicon in HL-60 cell line.

PO-117

EVALUATION OF FACTOR V LEIDEN, FACTOR II MUTATIONS AND MTHFR GENE HOMOZYGOUS MUTATION IN PATIENTS WITH DEEP VEIN THROMBOSIS, PULMONARY THROMBOEMBOLISM AND RETINAL VEIN OCCLUSION

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Background. During the last few years interest in laboratory tests for thrombophilia has been growing. Results are sometimes discordant, and impact on clinical practice is not well defined yet, especially for heterozygous mutations.

Methods - We evaluated heterozygous and homozygous Factor V Leiden (FVL) and prothrombin (FII) mutations and homozygous C677T mutation of methylenetetrahydrofolate reductase (MTHFR) gene in a cohort of 79 patients with deep vein thrombosis of the legs (DVT), 46 pulmonary thromboembolism (PTE) and 24 retinal venous occlusion (RVO). We also distinguished idiopathic forms from those secondary to malignancy, surgery, forced immobilization or hormonal therapy . *Results*. In 49% of patients with DVT, at least one of the three mutations is present. Mutations are more frequent in idiopathic forms than in secondary thrombosis (61,7% *vs* 30,5% p=0,0083) In 41% of patients with PTE, at least one of the three mutations examined is present: 36,3% of idiopathic forms and 48,3% of secondary forms (p ns). In patients with secondary PTE, mutations are more frequent than in those with secondary DVT without subsequent PTE (57.6% *vs* 30.5 p- 0.049). We did not observe patients with RVO secondary to surgery or malignancy; 2 patients were receiving hormonal therapy. Thrombophilic mutations are present in 45.8% of patients with RVO, the homozygous mutation of MTHR gene being present in 33%. In this group, the ratio between homozygous and heterozygous MTHFR mutation is greater than DVT group (0.88 *vs.* 0.42). *Discussion and Conclusions.* V Leiden factor, Factor II and MTHFR mutations are more frequent in idiopathic than in secondary DVT. Then, they probably play an important role in clinical practice. In the PTE group, mutations are more frequent in secondary forms than in idiopathic ones. So, they probably need the second event to generate PTE. However, they seem to play an active role in the progression from DVT to pulmonary thromboembolism. Although this is a retrospective analysis, we can hypothesize that a more intensive therapy could be required in patients with DVT and thrombofilic mutations. In patients with RVO it is interesting to note the absence of forms secondary to malignancy or surgery, maybe because local factors play an important role in pathogenesis. We need a prospective study in a larger cohort of patients to confirm these data.

PO-118

HUMAN MATURE BONE MARROW ADIPOCYTES MANTAIN THE SURVIVAL AND DIFFERENTIATION OF HEMATOPOIETIC STEM CELLS

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Introduction. Adipocytes are a cell population largely located in the human bone marrow cavity. In this specific microenvironment where adipocytes can interact with a variety of different cells, the role of fat is mainly unknown. The aim of this work is to molecularly and functionally characterize mature adipocytes isolated from human bone marrow (BM-A) and to study the interrelationship between BM-As and hematopioetic stem cells. *Methods.* BM-A were isolated after collagenase digestion and filtration. We studied the morphology of BM-A, their gene expression and immunophenotypic profile and their functional ability in the hematopoietic microenvironment, comparing them with adipocytes derived from adipose tissue (AT-A). *Results.* BM-A supported the survival and differentiation of hematopoietic stem cells in long-term culture demonstrating to be stromal cells with the same hematopoietic supporting function of AT-derived mesenchymal stem cells and AT-As. This ability is certainly linked to their role in the maintenance of the bone marrow microenvironment. BM-A showed a unilocular lipid morphology similar to AT-A and did not lose their morphology in culture; they showed a comparable pattern of stem cell-surface antigens to AT-A. In line with these observations, molecular data showed that BM-A expressed some embryonic stem cells genes, such as Oct4, KLf4, c-myc, Gata4, Tbx1, and Sox17, whereas they did not express the stem cell markers Sox2 and Nanog. Moreover, BM-A had long telomeres that were similar to bone marrow mesenchymal stem cells. *Conclusions*. Depending on specific demands, BM-A may acquire different functions based on their local environment.

PO-119

THE NUMBER OF CIRCULATING Th17 AND MYELOID DENDRITIC CELLS IS REDUCED IN PATIENTS WITH MYELOFIBROSIS

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Introduction. Myelofibrosis (MF) is a clonal disorder that may be preceded or associated with serious autoimmune or chronic inflammatory diseases, including Chron's Disease. Additionally, MF is burdened by a high rate of potentially life-threatening infectious complications. The issue of recurrent and opportunistic infections is increasingly relevant after the introduction in clinical practice of JAK-Inhibitors with immunosuppressive activity. Despite a dysregulation of the immune system seems to be a biological and clinical marker of MF, crucial subsets of the immune system are still poorly characterized. Here we investigated the phenotype/function of selected immune cells and to what extent these are deregulated in MF. In the immunological homeostatic process, regulatory T cells (Tregs) maintain tolerance by preventing autoimmunity and chronic inflammation, while IL-17-producing cells (Th17) are key to the pathogenesis of autoimmune diseases and protection against infections; key role in stimulating T lymphocyte proliferation and activation is played by circulating dendritic cells (DCs). *Methods.* We enumerated Th17 cells, Tregs and DCs in the peripheral blood (PB) of MF patients with JAK2V617F mutation (7 cases) and healthy controls (HC; 4 cases) by flow cytometry. Th17 cells were considered as CD4+CD161+CD196+ cells while Tregs as CD4+CD25high-CD127low cells. Two subpopulations of circulating DCs, myeloid CD11c+ and plasmacytoid CD123+ cells, were enumerated as well. After magnetic selection, we tested both phenotype of circulating monocytes and their capacity to differentiate in CD14-derived immature and mature DCs, using a specific cytokines cocktail. In addition, we tested the *in vitro* suppression activity of circulating Tregs using a mixed leukocyte reaction in which different amount of Tregs were cultured in the presence of T responders cells. *Results.* FACS analysis showed the same amount of circulating Tregs between HC and patients (29,8±5,6 Vs 30,4±15,2 cells/μL) as well as suppressive ability. However, a lower percentage (6,6%±1,5 Vs 12%±5) and number of circulating Th17 (13,08 ±7,22 Vs 93±39 cells/μL) was found. Interestingly, MF patients had 10-times lower percentage of plasmacytoid and myeloid DC compared to HC. Since monocytes are DC precursors, alterations in their differentiation may contribute to defective immune responses. Monocytes from MF had a different phenotype, compared to HC, characterised by higher levels of CD86, CD80 and chemokine receptor type 7 (CCR7). Immature DCs were CD14-positive, suggesting a different differentiation process. *Conclusions.* MF patients are characterized by specific quantitative and qualitative anomalies in immune cells. Decreased circulating Th17 and DCs, together with hyperactivated monocytes and monocytes-derived DCs, may play a role in the increased susceptibility to infections. Also, these data suggest an unusual behaviour of these cells in the context of the inflammatory network of MF.

PO-120

MANAGEMENT OF ACQUIRED HEMOPHILIA IN ELDERLY PATIENTS WITH NON HODGKIN'S LYMPHOMA

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Acquired hemophilia is a rare disorder characterized by autoantibodies against circulating coagulation factors. In this work, we describe our experience with three cases of indolent NHL with isolated prolonged aPTT and PT. Case 1: A 72 y.o. man referred to our Institution because of recurrent epistaxis and abnormalities of coagulation tests PT INR 2.5, aPTT ratio 2.73. No history of bleeding disorders, recent surgery or new drug intake were reported. Spleen enlargement and pancytopenia were observed. Moreover, it was present a reduction of Factor VIII, II, V, VII, IX, X and XI activity and positivity of antibodies against coagulation factors. Lymphoid infiltrates were observed in bone marrow examination. FNAB in the supraclavear lymphonode, performed after an injection of 60 μg/Kg recombinant Factor VIIa enabled the NHL diagnosis. The patient underwent to six administrations of chemotherapy with CEOP and achieved complete remission with coagulation parameters and factor activity normalization. Then, during followup, 11 months after the end of frontline treatment, the patient came to our Department for the onset of abdominal bleeding and relapse of the lymphoproliferative disease. After surgical treatment, the patient underwent to R-FN x 6 with CR. After the end of treatment, he underwent to Rituximab-based maintenance with persistence of normality of coagulation and factor activity parameters. Case 2: A 62 y.o. woman came to our observation for lymphoadenopathy, hepatoplenomegaly, anemia and lymphocitosis. She didn't present any personal or familial history of bleeding disorders and didn't take any drug. Laboratory tests showed: PT INR 3.26, aPTT ratio 4.92; there was also a reduction of Factor VIII, II, VII, IX, X and XI activity and appearance of antibodies against many coagulation factors. PET/CT revealed increased uptake at axillary and inguinal lymphonodes and spleen. Bone marrow analysis showed a lymphoid infiltrate and enabled the diagnosis of NHL. The patient underwent to six courses of R-Fludarabine, and after the second administration it was observed a PT and aPTT normalization. Case 3: A 80 y.o. man came to our observation because of pancytopenia and abnormalitites of coagulation tests: PT INR 2.28, aPTT ratio 3.2. No story of previous bleeding disorders. The activity of various coagulation factors was reduced: FII 54% FV 68% FX 60%. Bone marrow examination enabled the NHL diagnosis. The patient underwent to R-Chlorambucil courses, after which a normalization of PT and PTT was observed. All patients of this study underwent to Rituximab-based maintenance treatment to prevent the emergence of lymphoproliferative clone. The onset of an acquired coagulation disorder can be an useful diagnostic and progonostic marker of immunological impairment due to an underlying lymphoprolipherative disease. In particular, maintenance treatment may actively control the neoplastic clone reducing the risk of bleeding.

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COST-EFFECTIVE ANALYSIS OF PROPHYLAXIS WITH LAVIVUDINE FOR THE PREVENTION OF REACTIVATION IN OCCULT HEPATITIS B (OBI) IN PATIENTS WITH NON HODGKIN LYMPHOMA CD20+ UNDERGOING CHEMOTHERAPY WITH AND WITHOUT RITUXIMAB

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Occult HBV infection (OBI) is defined by the persistence of HBV in the liver without serum HBsAg and HBV-DNA. It represents a life threatening event during immunosuppressive chemotherapies. An OBI occurs in approximately 18% of HBcAb+ patients. International guidelines suggest surveillance for HBV markers in immunosuppressed patients, in particular monoclonal antibodies. In our study, the prevalence of OBI reactivation in Non-Hodgkin Lymphoma (NHL), in 498 NHL patients in our centre of Southern Italy, was 10.42% in HBcAb+ HBsAb- patients. In this work, we want to perform a cost-effectiveness analysis regarding the use of Lamivudine for the prevention of reactivation in OBI in patients with Non-Hodgkin Lymphoma undergoing chemotherapy with or without Rituximab. In fact, considering guidelines and literature, universal prophylaxis should have been applied to all HBcAb positive HBsAg negative patient. A cost-benefit issue arises: is it more cost-effective to treat all the HBcAb positive HBsAg negative patients with Lamivudine to prevent the OBI reactivation occurrence in a small quote of them, or may it be more effective a "wait and see" protocol? Our idea was to perform a cost-effectiveness analysis, comparing the costs of prophylaxis of an eventual HBV reactivation and the "monitoring" approach that was used in our patient based on the international guidelines. We calculated the cost of prophylaxis with Lamivudine in a time interval of twelve months, which encompasses the time of a standard Rituximab-containing chemotherapeutic protocol and a minimum time of follow-up. It has to be noticed that, very often, NHL patients need more than one chemotherapy cycle to obtain NHL remission, and, sometimes, if they do not obtain a complete remission, undergo to long-term "maintenance" treatments with Rituximab. These patients (HBcAb positive) are at high risk of HBV reactivations, due to long times of immunosuppression. Nevertheless, even if our calculations underestimated the costs of prophylaxis, the "monitoring approach" resulted cost- effective. Moreover, even though in our series no serious events in terms of morbidity and/or mortality occurred, in other literature reports a monitoring approach did not guarantee patients survival. These detrimental results could be ascribed to the delayed start of Lamivudine treatment if the monitoring is not adequately strict. Also, it has been reported that performing only the transaminase monitoring should not be acceptable to prevent severe reactivations. Our monitoring approach resulted efficacious probably because of the monthly ALT assay was strictly observed. We report an advantage in the "monitoring" approach even if, due to the retrospective nature of our study, we cannot draw any firm conclusion on which should be the best approach (universal prophylaxis *vs* monthly ALT monitoring). A randomized controlled trial might be needed to properly address this issue.

Table 1.

	Unitary Cost	n. patients	Total per patient	Duration [days]	Total
Cost of prophylaxis					
Lamivudine	€ 3,18	48	€ 152,64	360	€ 54.950,40
HBV DNA monitoring	€ 130,00	48	€ 6.240,00	6	€ 37.440,00
HBsAg monitoring	€ 17,00	48	€ 816,00	6	€ 4.896,00
AST/ALT monitoring	€ 5,74	48	€ 275,52	12	€ 3.306,24
Total	**€ 155,92**	**48**	**€ 7.484,16**		**€ 100.592,64**
Cost of HBV Reactivation					
HBV DNA monitoring	€ 130,00	48	€ 6.240,00	6	€ 37.440,00
AST/ALT monitoring	€ 5,74	48	€ 275,52	12	€ 3.306,24
Cost of DRG 205 [v24 Grouper]	€ 3.769,10	5	-	-	€ 18.845,50
Total	**€ 3.904,84**	-	-	-	**€ 40.746,24**

Drg 205: Liver disease except malignancies, cirrhosis, alcoholic hepatitis with cirrhosis.

BRENTUXIMAB VEDOTIN IN RELAPSED/REFRACTORY LYMPHOMA: OUR EXPERIENCE IN THE TREATMENT OF YOUNG PATIENTS

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In this work, we analyze a successful experience with brentuximab vedotin in the management of relapsed and refractory lymphoma. Case 1: N.A., 21 y.o. male patient, with diagnosis in November 2011 of Systemic anaplastic large-cell lymphoma (ALCL), Stage IIIB, non B non T, ALK-positive, CD30+. In November 2011 he underwent to CHOP/14 x 6 with CR. Then, he underwent to IEV, but, after 2 courses, the patient referred to our Institution for abdominal pain: a PET/CT was performed documenting a relapse. So, he switched to DHAP, with an apparent improvement, but, one month after the first course there was a worsening of clinical conditions. Then, for progressive disease, there was a switch to Brentuximab treatment. After first course, the patient went to Emergency Medicine for respiratory problems, and, after the solution, we continued the protocol. The treatment was well tolerated, and, after 6 courses, the patient is in CR and he undergoes, in November 2013, to Allogenic BMT. Now, after 4 months, the patient has no signs of disease. Case 2: T.A., 35 y.o. female patient, diagnosis in February 2011 of classic nodular sclerosis Hodgkin's Lymphoma, Stage IIB, with mediastinal bulky disease. Frontline treatment was ABVD, 12 courses, with the result of a PR. After, Radiotherapy was started on mediastinal mass, remaining in SD. After 3 months, there were new localizations documented from PET/CT and IGEV protocol was started, total of 3 courses, after which she underwent to AutoBMT in September 2012. PET/CT performed in February 2013 documented a progressive disease: Brentuximab was started and even if after 3 courses the patient was in SD, after 6 courses there was a PR, which became CR after 12 courses. The treatment was well tolerated, and the only documented side effect was alopecia. Case 3: G.R., a female 30 y.o., with diagnosis in April 2004 of Hodgkin Lymphoma, Stage IIB, with splenic localization. Frontline treatment was VEBEP, 11 courses, with the result of a PR. Then, Radiotherapy was started but a relapse of disease was documented in May 2005. Then, IGEV treatment was started, 3 courses and then she underwent to AutoBMT with a CR. In September 2006 PET/CT documented the second relapse: MOPP protocol was started (12 courses), until April 2007, with PR. In October 2007 a third relapse was documented, treated with Gemcitabine, with a SD. The patient underwent to DHAP-R, 4 courses, with the result of a PR. So, in October 2009, the patient started Rituximab maintenance, until July 2012, when it was interrupted for progression of disaese and bad conditions. In that moment she switched to Brentuximab and after 3 courses there was a nCR and after 6 courses she was in CR. The only side effect was a Grade 1 Neuropathy. In our experience, Brentuximab Vedotin can be considered an effective option for advanced patients, relapsed and refractory to almost all available therapeutic resources, and it could be considered as a bridge-treatment to AlloBMT.

SUPPORTIVE INTRAVENOUS IG (IVIG) TREATMENT IN RITUXIMAB-INDUCED HYPOGAMMAGLOBULINEMIA IN PATIENTS WITH NON HODGKIN LYMPHOMA

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Rituximab is a chimeric murine/human-engineered monoclonal antibody which can selectively deplete CD20-expressing B cells in peripheral blood and lymphoid tissues. This retrospective single center analysis, aims to evaluate the incidence of Rituximab-related hypogammaglobulinemia (hypoIg). In our institution, in patient affected by NHL in treatment with Rituximab-based regimens, we performed serial quantitative serum immunoglobulin (SIg) concentration at baseline, after chemotherapy, during and after Rituximab maintenance treatment. IgG, IgA and IgM deficit were respectively defined by level below 700 mg/dL, 70 mg/dL and 40 mg/dL. We considered patients as symptomatic if they developed at least two non-neutropenic infections in a 6-months observation period after or during Rituximab-based treatment. 88 patients with NHL and SIgG monitoring were retrospectively analyzed, 24% of them were relapsed or refractory after first courses of chemotherapy. The median age of patients was 61 years (range: 28-80 y). From histological examinations, the patient were diagnosed as follicular lymphoma (FL) (n=53), small lymphocytic lymphoma (SLL) (n=9), marginal zone lymphoma (ML) (n=11), mantle cell lymphoma (MCL) (n=9), diffuse large B-cell lymphoma (DLBCL) (n=6). Patients received a median of 11 administrations of Rituximab (range: 6-27). The median follow-up of surviving patients was 3.6 years. Before treatment with Rituximab, 9/88 (10.2%) had low SIgG levels (6 FL, 1 MCL, 1 SLL, 1ML) and in 4/9 (44.4%), during R-maintenance treatment, IVIG administra-

tion was necessary. After R-based chemotherapy regimens, IgG deficiency was observed in 20/88 (22.7%), no one needed IVIG, despite 7/20 (35%) were symptomatic. After or during Rituximab-based maintenance treatment, in 22/88 (25%) IgG deficiency was observed after a median of 10 R administrations; the deficit was observed in 77% (17/22) within the fourth R maintenance administration and in no one after the sixth R administration. In this category, 12/22 (54.5%) were symptomatic and 4/22 (18.2%) required IVIG supportive treatment. In all 8 patients who needed IVIG treatment, at least two different Ig isotypes were deficient. Rituximab treatment is associated with a high risk of hypoIg development. Moreover, the number of administrations is strictly related to the development of symptomatic hypoIg, and the risk of hypoIg increases in patients in Rituximab-based maintenance. The decision to introduce supportive treatment with IVIG in non-neutropenic patients was related to the number of infection episodes. HypoIg often is undiagnosed, in particular for the presence of confounding symptoms. Our study suggests that Ig levels monitoring and supportive IVIG treatment should be considered in this patients subset.

AN UNUSUAL CASE OF LIGHT CHAIN DISEASE IN REMISSION AFTER BONE MARROW TRANSPLANTATION

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This is a case report of a 31 years old woman with history of ulcerative colitis , actually in remission. In December 2012, the onset of acute renal failure and severe anemia with κ light chains paraproteinemia caused the patient to be admitted to UONephrology.The patient was subjected to transfusion therapy and dialysis with specific membranes in order to eliminate the excess of light chains (7 dialysis treatments with TheraLite). Since we suspected multiple myeloma /amyloidosis AL/ LCCD, bortez. 1.3 mg/m2 IV + dexamet. 20 mg was quickly initiated; 5 cycles were completed, reaching after the first normal renal function and FLC ratios. BM plasma cell immunophenotype was performed;[1] plasma cells were <1% and polyclonal, with a normal phenotype. Morphological examination of BM samples was conducted by May-Grünwald-Giemsa staining method of BM films.[2-3] Plasma cells were <5% and normal, as were the other maturative cell lines. Bone biopsy reveal 10-20% of mature plasma cell elements, light chains were not available. Renal biopsy demonstrated CAST Nephropathy with κ light chains. Fat cord examination showed the presence of rare κ chains. The differential diagnosis carried us to exclude the diagnosis of amyloidosis (fat biopsy was not conclusive) and MM (plasmacells infiltration was approximately 10%, with no signs of pathological phenotype and clonal restriction).The diagnostic difficulties motivated the private consultation with the BMT Centre of Pavia, following which the patient accepted the transplant program we proposed. Here we report results provided by Centre of Pavia: renal biopsy demonstrated interstitial fibrosis, in absence of amyloid fibrils or material immunoreactive for anti-κ, anti-Œª chains and anti-SAA antibodies. BM biopsy and periumbilical fat examination showed no evidence of amyloid deposits. In conclusion, the diagnosis of κ light chain disease is based on the absence of bone marrow plasma cell infiltration, not-confirmation of the presence of amyloid material . Given the young age, we have proposed the patient to perform the mobilization of PBSC for a subsequent transplant approach .On 08.10.2013 we collected a total of 60 x 10^6. In 12.11.13, the first bone marrow transplantation (Melphalan 140 mg / DT 220 mg in 2 days) was characterized by WHO grade 2 mucositis, with Neutrophils> 500 µl at day +9, Platelets> 20,000 per µl at 10+ day. Six month after transplantation, the patient shows completely normal renal function, with no signs of disease at BM examination and FLC ratio within the reference range.

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AN UNUSUAL CASE OF ACUTE MYELOID LEUKEMIA WITH SPLENOMEGALY, EOSINOPHILIA AND RUNX1-MECOM FUSION TRANSCRIPTS

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Introduction. The t(3;21)(q26;q22), resulting in the fusion transcript RUNX1-MECOM (formerly AML1-MDS1-EVI1), is a rare cytogenetic abnormality, with poor prognosis, reported in approximately 1% of all MDS or AML cases and mainly occurring in therapy-related myeloid neoplasms, in accelerated or blast phase of CML or other myeloproliferative neoplasms (MPN), and, rarely, in *de novo* AML. *Methods*. A 63-year old man was admitted in April 2013 because of splenomegaly and persistently increased LDH, since 2009. Complete blood count (CBC) was normal and neither abnormal myeloid cells nor atypical lymphocytes were detected by morphologic and flow cytometry analyses on PB samples. Neither BM examination nor cytogenetic and molecular analyses were performed at this time-point. Four months later, the patient was subsequently admitted because of fever, fatigue, abdominal pain and drenching night sweats. CBC revealed WBC count 12.2 x 10^9/L with 43% eosinophils and 18% blasts, Hb 9.7 g/dl and Plt count 17 x 10^9/L. *Results*. The morphologic examination of BM aspirate and trephine biopsy showed hypercellularity, with marked proliferation of eosinophil granulocytopoiesis, multilineage myelodysplastic features and a blast cell count 20-30%. Mild BM fibrosis was documented. AML with MDS-related changes and eosinophilia was thus diagnosed. Conventional G-banding showed 46,XY,t(3;21)(q26;q22) karyotype. Molecular examinations, namely RT-PCR and subsequent sequencing analyses performed on both PB and BM samples, also documented RUNX1-MECOM alternatively spliced multiple fusion transcripts. Further FISH and molecular studies failed to detect either BCR-ABL, PDGFRA, PDGFRB and FGFR1 rearrangements or NPM1 and FLT3 mutations, whereas c-KIT D816V and JAK2 V617F mutations were found on PB and BM samples. The patient was refractory to remission induction and subsequent salvage chemotherapy. *Discussion*. This is the first case of AML with RUNX1-MECOM fusion transcript, showing marked peripheral and BM eosinophilia. The patient was previously observed because of persisting homogeneous splenomegaly and increased LDH, which, in the absence of any other features suspected for hematologic malignancy, were initially attributed to both minor β-thalassemia and previous acute myocardial infarction, occurred in 2009. The retrospective analysis of the clinical features combined with the documentation of both JAK2 V617F and c-KIT D816V mutations at AML diagnosis may suggest an aggressive leukemic transformation with eosinophilia and t(3;21)(q26;q22) translocation of a previously unrecognized MPN, rather than the occurrence of a *de novo* AML. Either BM morphologic examinations or cytogenetic/molecular analyses, at least on PB samples, may be useful to rule out MPN in similar cases observed for persisting/worsening splenomegaly and increased LDH, even in the absence of significant morphologic and immunophenotypic abnormalities on PB.

PRIMARY LYMPHOMA OF THE PROSTATE TREATED WITH RITUXIMAB AND BENDAMUSTINE: A CASE REPORT AND REVIEW OF THE LITERATURE

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Introduction. Primary lymphomas of the prostate are rare and incidentally found following prostate surgery. The authors report the case of low grade non-Hodgkin lymphoma of the prostate managed with association of rituximab and bendamustine. *Discussion*. One third of non-Hodgkin lymphomas occur in extranodal sites. Primary lymphoma of the prostate is very rare and occurs in men aged 60 years in average: it represents 0.09% of prostate tumors and 0.1% of all

non-Hogdkin lymphomas. Secondary involvement of the gland is the most common presentation in such cases. The most frequent presentation forms are obstructive urinary symptoms. The occurrence of systemic symptoms or changes in laboratory tests are uncommon. The digital rectal examination usually reveal an extremely enlarged prostate with normal consistency, with normal PSA. The definition of primary lymphoma of the prostate is based in several criteria. Chemotherapy constitutes the primary therapeutic approach. *Case report*. A 60 year-old man was admitted to our hospital having signs and symptoms suggestive of prostatic disease. Digital rectal examination showed a voluminous prostate with fibro-elastic consistency. An ultrasonography of the urinary tract showed an enlarged prostate. Blood tests revealed normal serum tumor marker PSA and serum creatinine. The TC scan and PET total body evidenced a voluminous expansive lesion exclusive of the prostate and absence of lymph nodal involvement. Histological and immunocytochemical studies of the biopsy of the prostate showed low grde B-cell non Hodgkin lymphoma CD-20+. The diagnosis of primary extra nodal lymphoma of the prostate at the stage IAE was performed. The patient was managed by association of rituximab 375 mg/mq and bendamustine 90 mg/mq. *Conclusion*s. Malignant lymphoma involving the prostate is extremely rare; according to the literature, the treatment and prognosis is the same as that of other nodal lymphomas. Systemic chemotherapy , with rituximab - based regimen, represents the initial and preferential therapeutic method. Prognosis is related to the patient age, histologic type, and treatment or clinical stage of the disease at presentation.

BLASTIC PLASMACYTOID DENDRITIC CELL NEOPLASM (BPDCN) TREATED WITH SUCCESSFUL WITH VNCOP – B CHEMOTHERAPY PROTOCOL

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Introduction. BPDCN neoplasm is a rare and highly aggressive hematological malignancy derived from precursors of plasmacytoid dendritic cells that recently it is classified among "acute myeloid leukemia and related precursor neoplasms" in the 2008 WHO classification. Discussion. BPDCN predominantly affects males generally occurs in the elderly and the incidence in the general population is extremely low, accounting for 0.44% of all hematologic malignancies and 0.7% of cutaneous lymphomas; the leukemic form of disease is a rare phenomenon, representing <1% of cases of acute leukemia. BPDCN has an aggressive course and typically presents with cutaneous involvement as the first manifestation, with subsequent or simultaneous spread to bone marrow and peripheral blood. An accurate diagnosis of BPDCN is essential in order to provide treatment promptly, especially considering that the initial clinical presentation is often indolent. Despite an initial response to systemic chemotherapy, the disease regularly relapses and the median overall survival is dismal. Case report. Our patient is a 80 year old female who presented at the time of diagnosis multiple skin lesion and lymph node enlargement. Skin biopsy revealed a diffuse infiltration of lymphoid cells positive for CD4 and CD56, indicating a diagnosis of BPDCN. A subsequent bone marrow aspirate and biopsy and a lymph node biopsy confirmed a diagnosis of BPDCN. The patient was classified as stage IVA involving the skin, multiple lymph nodes and the bone marrow. Peripheral blood examination showed pancytopenia without evidence of blasts. Due to the scarsity of data and lack of consensus as to the optimal treatment approach for BPDCN and in consideration of the performance of the patient, she was treated with a regimen called VNCOP-B . After one cycle the bone marrow biopsy showed a partial response greater than 50% reduction in blast percentage and with complete response of lymph node and lesion of the skin. Actually the patient is in therapy with a second cycle VNCOP-B with stable disease and in absence of skin lesions. *Conclusions*. BPDCN is a rare disease. The prognosis of BPDCN is poor in most cases with rapid progression despite administering chemotherapy. Its diagnosis is not easy and requires expert physicians, both clinicians and histopathologists. Effective therapies have not been established yet. This case is significant because has been treated, with a good response, with a protocol used for NHL of old patients.

A CHRONIC MYELOID LEUKEMIA PATIENT WITH ATYPICAL KARYOTYPE AND A BCR-ABL FUSION VARIANT CAUSED BY A THREE WAY CHROMOSOME TRANSLOCATION

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Introduction. The objective of this work was to confirm the presence of complex translocation in a rare BCR-ABL fusion variant in a CML patient positive for t(9;22) translocation but negative for common major and minor breakpoint cluster regions. *Methods.* Bone marrow cells from a patient with elevated white blood cell count, splenomegaly and hepatomegaly was subjected to classical cytogenetics, FISH with two types of probes and RT-PCR using commercial kits. *Results.* The translocation event between chromosomes 9 and 22 could be successfully detected. BCR/ABL ES dual color translocation probe generated a classical balanced translocation within the interphase cells and presented a '2O1G1F' signal pattern. Same situation was seen on metaphases, but only one orange signal was seen at telomeric region of chromosome 9 while the other one was in a centromeric region of a chromosome that supposedly belongs to A group (Figure 1). BCR/ABL dual color, dual fusion probe generated within the interphase cells a '2O2G1F' signal pattern. BCR-ABL transcripts detection by qRT-PCR failed to generate any signal using probes from commercial kits to detect common breakpoints within the M- and m-BCR regions. Classical cytogenetics revels the presence of Philadelphia chromosome and a further translocation involving a chromosome of A group, presumably chromosome 2. *Conclusions.* The CML atypical karyotype was caused by complex translocation. Fluorescence *in situ* hybridization analysis of metaphases and classical cytogenetics lead to a more precise cytogenetical characterization, so it is necessary to perform a whole-chromosome painting to determine the chromosome involved in this translocation. This case bring to the attention the need to investigate atypical BCR-ABL fusion gene variants when common breakpoint cluster regions are missing or mutate, so that minimal residual disease (MRD), critical for disease monitoring, can be performed to avoid false positive remission cases. It also emphasizes the utility and significance of cytogenetics and FISH techniques in primary diagnosis of CML.

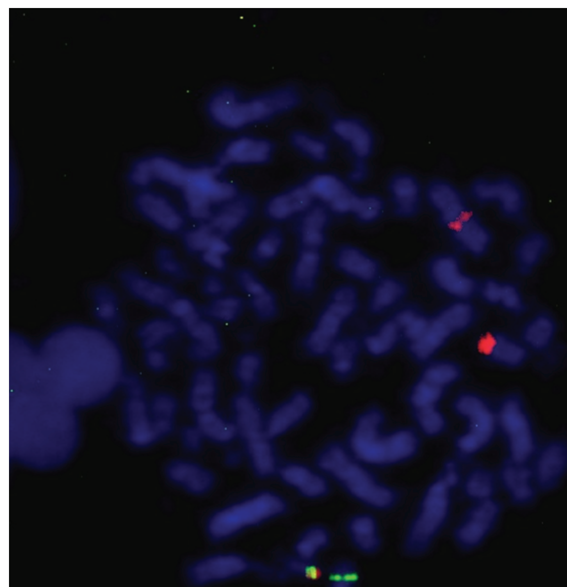

Figure 1.

SEARCHING FOR WT1 MUTATIONS: IDENTIFICATION OF A NOVEL VARIANT IN A PATIENT WITH ACUTE MYELOID LEUKEMIA

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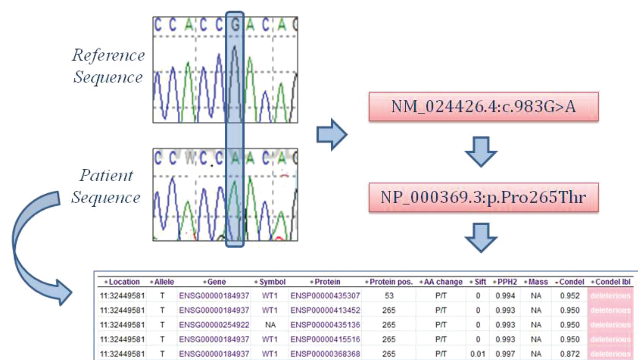
Introduction. With the availability of the human genome sequence, molecular abnormalities impact on diseases became a significant focus of

many researchers.[1,2] The role of Wilms' tumor1 (WT1) gene in AML is not completely understood. It is over-expressed in AML and used as minimal residual disease (MRD) marker. Gene mutations are found in approximately 10% of AML patients with hotspots in zinc-finger domains on exons 7 and 9, but their clinical impact is still unclear.[3,4] The presence of novel WT1 variants could affect its role in predicting cancer progression. *Methods.* Patient gDNA was isolated at disease onset from the bone marrow (BM) of a 53 years-old man with M0 AML. CBC revealed: Hb 7.3 g/dL, WBC 54,5x10e3/ul, PLT 28x10e3/ul with 96% blasts, 2% neutrophils and 2% lymphocytes. He had enlarged submandibular and laterocervical lymphnodes but not liver or spleen enlargement. BM examination showed 90% blasts. Cytogenetic analysis revealed a karyotype 46,XY,del(11)(p13pter) in 8/15 metaphases. At flow cytometry evaluation blast cells were MPO-/+, CD34+, CD117+, CD33+, TdT-, HLA DR-/+, CD3-, CD13+, CD16-, CD56-, CD19-. Molecular biology was negative for NPM1 mutations, FLT3-ITD, AML1-ETO, INV16. WT1 copies were 58.5 (cut off level: 250 copies). After induction therapy (ARA-C and Daunorubicin: 3+7), the patient obtained the CR1. Flow cytometric MRD was negative MRD: 0.01%. WT1 levels were persistently low (45.8 copies). The patient underwent ASCT and, after six months, he relapsed with 17.1 WT1 copies. He received II line chemotherapy (FLAG-Ida) but he died at day +19, during chemotherapy induced aplasia. Due to the low WT1 expression, we also investigated all gene regions by performing PCR with specific primers and gene sequencing. The similarity between our fragments and WT1 RefSeq (GenBankID: NM_024426.4) was checked by means of the BlastN algorithm (NCBI database) and the Mutation Surveyor software. The variant deleteriousness was assayed by using Condel server. *Results.* We found a novel, not previously reported variant (NM_024426.4:c.983C>A, HGSV nomenclature) that determines, at protein level, the substitution of proline, a hydrophobic aminoacid, with threonine, a hydrophilic aminoacid (NP_000369.3:p.Pro265Thr, HGSV nomenclature). Consequently protein chemical and physical features are significantly affected. The Condel server output showed that the mutation is a pathogenetic substitution. These aspects suggest a functional impairment of WT1 protein that could affect its role: maybe because of this, we observed a low gene expression in our patient (Figure 1).



Location	Allele	Gene	Symbol	Protein	Protein pos.	AA change	Sift	PPH2	Mass	Condel	Condel lbl
11:32449581	T	ENSG00000184937	WT1	ENSP00000435307	53	P/T	0	0.994	NA	0.952	deleterious
11:32449581	T	ENSG00000184937	WT1	ENSP00000413452	265	P/T	0	0.993	NA	0.950	deleterious
11:32449581	T	ENSG00000254922	NA	ENSP00000435136	265	P/T	0	0.993	NA	0.950	deleterious
11:32449581	T	ENSG00000184937	WT1	ENSP00000415516	265	P/T	0	0.993	NA	0.950	deleterious
11:32449581	T	ENSG00000184937	WT1	ENSP00000368368	265	P/T	0.01	0.997	NA	0.872	deleterious

Figure 1.

Conclusions. The novel variant observed could be responsible for low WT1 expression. The role of WT1 as oncogene is maintained but the presence of our codon change could explain the lack of correlation between gene expression and clinical outcome.

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A NEW THERAPY VIRUS-RELATED FOR B-CLL?

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Introduction. The chronic lymphocytic leukemia is the more frequent type of leukemia present in the west countries. The immunoglobulin gene repertoire by CLL malignant B cells displayes frequently the existence of subsets with quasi-identical (stereotyped) B-cell receptors (BCRs), implying the recognition of strutturaly similar epitops, likely selecting the leukemic clone. Rituximab is a monoclonal chimeric antibody anti-CD20, already used in B-lymphomas and also in chronic lymphocytic leukemia, often in association with chemotherapy. The author displays a clinical case of a man 78 years old, affected by chronic lymphocytic leukemia to B-cells CD5+, that achieved a clinical remission after immunological treatment. *Methods*. The schedule of therapy has been administred in this way: first day IgG anti-Cytomegalovirus at the dose of 5000 U in 1 hour of i.v. infusion, second day Rituximab at the dose of 375 mg/m2 (to first cycle and then 500 mg/m2) in slow i.v. infusion (3 hours), thirth day Valgancyclovir 450 mg twice a day for 15 days at alternate cycle. If wasn't evidence of neutropenia,the treatment continued for 5 cycles. *Results*. Since the first cycle, the blood count showed a clean decrease of lymphocytosis that, from initial value of 55000/mm3 halved to 22000/mm3 together with a sensible reduction of lymphoadenopathies. After the fourth cycle, it displayed a value of WBC of 3400/mm3 (N 2100/mm3, L 1000/mm3) with disappearance of CD5/CD19 leukemic clone to cytofluorimetric exam and normalization of CD19/k, CD19/lambda ratio. Already, in previous years, we have treated some patient affected by CLL with only Rituximab every 15 days for 5 or 6 cycles, achieving a clean decrease of lymphocytosis and in some case a clinical remission of disease. The difference, in comparison with last patient, was the speed with which it was obtained the decrease of lymphocytosis. *Conclusions*. On the basys of this case and few other subjects treated, we can't draw any absolute indication of sure clinical significance for the future tratment of this leukemia. However, for my opinion, it's important to underline as this treatment doesn't make use of traditional chemotherapy with a less impact of side effects and hence more tolered. Furthermore, in order to speculative point about of pathogenesis of the illness, some researchers as Steininger C and Stamatopoulos K have found a relationship between stereotyped VH-regions of Immunoglobulines (VH4-34, VH1-69) and antigenical stimulation by cytomegalovirus and Epstein Barr virus. The consequence could be the proliferation of leukemic clone stimulated by specific epitops of antigen. In this case the role of membrane antigen CD20 is decisive, because this marker is a calcium-channel and permit the entry of calcium ions in the cell, with start of the signal of proliferation, instead of apoptosis pathway. The first day cytomegalovirus-antibodies were infused as antigen that link leukemic cells and second day rituximab block the proliferation.

A FATAL HEMORRHAGIC EVENT IN MULTIPLE MYELOMA

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Introduction. Several mechanisms of bleeding in patients with multiple myeloma are known: thrombocytopenia, paraprotein-related mechanisms, platelet dysfunction. A 49-year-old female died during induction therapy of IgG k, BJ positive MM because of a fatal hemorrhagic event. She had had an episode of metrorrhagia, six months before first admission to our Unit. *Methods.* At diagnosis she had a diffuse bone marrow plasmacellular infiltration, mild normocromic normocytic anemia and a normal coagulation profile; 24 hours proteinuria: 425 mg/L. Total body X Rays: fracture of X right rib. Cytogenetics: amplification of 1q21 region on chromosomes 1 and 8, delection of chromosome 13. Ecocardiography negative for amyloidotic cardiomyopathy. The patient was given two 28 days courses of induction therapy consisting of bortezomib sc (1.3 mg/mq) on days 1,4,8,11, cyclophosphamide 500 mg/mq on days 1 and 8, dexamethasone 40 mg on days 1,2,4,5,8,9,11,12. Between first and second course of therapy the patient reported iatrogenic cutaneous reaction, resolved with antihistamines and prednisone, after stopping oral therapy. At the start of the third cycle, delayed because of the onset of dyspnea and dry cough, not associated with fever: drastic decrease of paraprotein (5.95 *vs* 0.35 g/dL), persistence of mild anemia and platelet count at superior limits. Therapy was regularly administered on days 1 and 4 of the third cycle. Vital signs on days of therapy were normal. RESULTS: Two days after last administration, she reported a severe headache and right otalgia, irresponsive to paracetamol and ibuprofen; the patient was awake, without neurological abnormalities; she presented vomit not preceded by nausea; she was bradicardic and had an elevated systolic pressure. Platelet count and coagulation tests were normal. On CT scan an atypical intracranial hemorrhage was recognized in right temporo-parietal region, surrounded by an hypodense area of edema. The day after admission to Stroke

Unit the level of consciousness suddenly decreased; a second cerebral CT showed massive enlargement of hemorrhagic area and edema. Neurosurgery was not performed because of severity of this setting. The patient died after 12 hours into resuscitation room. *Conclusions.* Occurrence of this hemorrhagic fatal event made we wonder about multiple aspects: was it simply due to a sequence of hypertensive crises, facilitated during steroid therapy? Can I hypothesize abnormalities of FXIII, not explored by conventional coagulation tests? Is this the case of acquired platelet dysfunction? Was there an amyloidotic deposition on cerebral vessel walls, even if laboratory testing were not suggestive for this setting? May the patient have an unrecognized systemic vasculitis, in absence of signs of involvement of other districts? Could it be the unusual expression of idiosyncrasy to bortezomib? Is this therapy as safe as it was thought to be? Autopsy could be useful but it was not available.

IMAGING ECOGRAFICO NELLA DIAGNOSI DI MIELOMA PANCREATICO

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Introduction. Extramedullary multiple myeloma (MM) represents an atypical presentation of the disease, accounting for 4-16% of cases at diagnosis and 6-20% at relapse. We describe a case of pancreatic localization at relapse. *Methods*. A 52-year old woman complaining for bone pain and fatigue was diagnosed as IgG K MM, III A according to Durie et Salmon, with cytogenetical finding of translocation 11;14. At diagnosis, serum monoclonal component was 11% and urinary k chains were 880 mg/L; bone marrow plasma cells were 40%. Multiple osteolysis were detected by conventional radiography. The patient received four cycles of lenalidomide plus dexamethasone, obtaining a very good partial response. She was offered staminal cell autologue transplantation, but refused and was followed-up without treatment. She relapsed after 13 months, and received 3 courses of thalidomide plus dexamethasone, with a final partial response, followed by reinfusion of peripheral staminal cells. After a 15-month follow-up, the patient developed severe hyperglycemia (480 mg/dL) and oedema of the left leg. An abdominal ultrasound showed the presence of a 5,2x4,1 cm mass in the pancreatic tail (Figure 1).

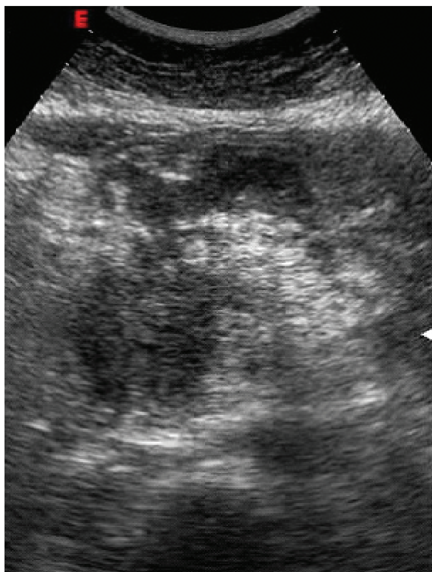

Figure 1. Pancreatic myeloma visualized as a hypoechogenic area.

CT scan showed the presence of massive adenopathies in the left iliac region, involving left ureter, ileopsoas and gluteus. The patient underwent contrast-enhancement-US guided biopsy of the pancreatic mass, and bone marrow aspiration. Both specimens allowed diagnosis of MM relapse. *Results*. Patient started treatment with bendamustine, bortezomib and dexamethasone, but the first cycle was followed by signs of rapid progression of the lymphoadenopathies. Radiotherapy was started in an attempt to reduce the leg lymphedema which caused skin ulcerations and fluid leakage. *Conclusions*. Extramedullary myeloma disease (EMD) is a rare entity. The most frequent site is the upper respiratory tract, but gastrointestinal localizations have also been described. It has been largely reported that EMD is associated with a worse performance status and survival. Response to therapy is generally poor, with a high rate of relapse, progression and death. There is no consensus on treatment. It's really important to respect dose intensity and density, in attempt to reduce disease progression and to maintain response. Pancreas involvement incidence has been estimated to be 2-3%. Most frequent symptoms are jaundice and abdominal pain. CT and NMR scan are the gold standard imaging techniques, but contrast-guided US may be helpful for a rapid diagnosis. Indeed, eco-guided biopsies of pancreatic lesions can be rapidly obtained with a very low rate of procedure-related complications, thus allowing a rapid histologic study. In our opinion, a multidisciplinary collaboration between haematologist, radiologist and cytologist is essential in order to obtain the diagnosis as soon as possible.

CONTRAST – ENHANCEMENT ULTRASOUND GUIDED BIOPSY IN DIAGNOSIS OF PANCREATIC MYELOMA

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Introduction. Extramedullary myeloma disease (EMD) represents an atypical presentation of the disease, accounting for 4-16% of cases at diagnosis and 6-20% at relapse. We describe a case of pancreatic localization at diagnosis. *Methods*. A 46-year old man with a history of diabetes mellitus was diagnosed with IgA lambda multyple myeloma (MM), stage III B according to Durie et Salmon, with deletion of chromosome 13p and translocation (4;14). Monoclonal component was 1,16 g/dl, urinary Bence-Jones 1 g/L, bone marrow plasma cells were 50%. He was treated with an association of three drugs (bortezomib, liposomal anthracycline and dexamethasone) obtaining, after 5 cycles, a very good partial response (VGPR). After mobilization and cryopreservation of peripheral blood stem cells, he underwent autologous stem cell transplantation (ASCT) and obtained the complete remission. After 12 months,the patient relapsed with multiple osteolytic lesions associated to elevation of monoclonal component (1,2 g/dL) and started monthly courses of lenalidomide and desametasone. After 3 months, he developed jaundice with transaminase and bilirubin elevation . An abdominal ultrasound showed a 5 cm hypoechoic mass in pancreatic tail (Figure 1), causing biliary tree compression. NMR confirmed the pancreatic involvement; Fludeoxyglucose with PET/CT (FDG-PET/CT) showed multiple active osteolytic lesions. Contrast-enhancement ultrasound guided biopsy of the pancreatic mass allowed diagnosis of myeloma.

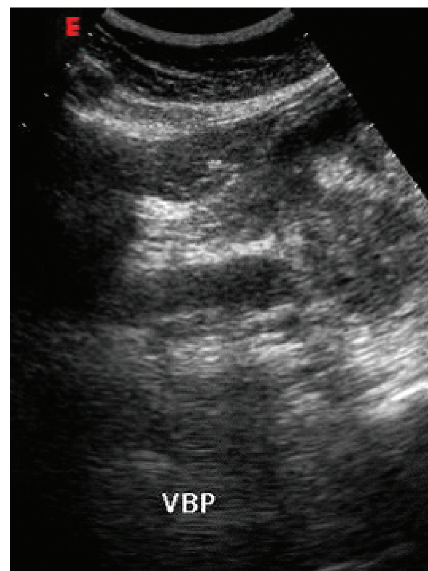

Figure 1. Pancreatic myeloma visualized as a hypoechogenic area.

Results. The patient received treatment with bendamustine plus bortezomib and dexamethasone, but further enlargement of the pancreatic mass prompted to perform a different therapeutical association based on lyposomal anthracycline, cyclophosphamide and dexamethasone. *Conclusions*. Extramedullary myeloma disease (EMD) is a rare entity. The

most frequent site is the upper respiratory tract, but gastrointestinal localizations have also been described. It has been largely reported that EMD is associated with a worse performance status and survival. Response to therapy is generally poor, with a high rate of relapse, progression and death. Pancreas involvement incidence has been estimated to be 2-3%. Most frequent symptoms are jaundice and abdominal pain. TC and NMR scan are the gold standard imaging techniques, but contrast-guided US may be helpful for a rapid diagnosis. Indeed, eco-guided biopsies of pancreatic lesions can be rapidly obtained with a very low rate of complications procedure-related, thus allowing a quick histologic study.

FATAL OUTCOME OF A PATIENT WITH PAROXYSMAL NOCTURNAL HAEMOGLOBINURIA IN THE ABSENCE OF TREATMENT WITH ECULIZUMAB

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Introduction. Paroxysmal Nocturnal Haemoglobinuria (PNH) is a rare acquired clonal disorder of hematopoietic stem cells related to a somatic mutation in the PIG-A gene that involves all blood cells..The development and successful clinical trial of a humanized monoclonal antibody against the terminal complement protein C5 (Eculizumab) has improved the quality of life for patients with PNH by reducing hemolysis, thromboembolic events (TE), and transfusion requirements. Often TE represents the most frequent cause of mortality. Thrombosis in PNH patients frequently follows episodes of acute hemolysis suggesting that inadequate complement inhibition or hemolysis might be an important underlying factor contributing to the thrombophilic condition seen in these patients. Description of case. A 40-years old male patient with Multiple Sclerosis and suspected bone marrow ipoplasia was admitted at the hospital for upper gastrointestinal hemorrhage from esophageal varices, porto-mesenteric thrombosis of the splenic and hepatic veins. Imaging findings were compatible with the diagnosis of Budd Chiari syndrome. Clinicals findings: pallor and hepatosplenomegaly. Laboratory findings: WBC 3660/mmc; Hb 7.8 gr/dl; Ht 24%; MCV 70fl; PLT 79000/mmc. Reticulocytes 4%, haptoglobin30 mg/dl. High LDH levels (637 UI/l), elevated liver enzymes, ferritin 10 ng/ml, glucose, urea creatintine, electrolytes normal, direct Coombs negative. Antithrombin, Protein C and Protein S in normal range. Factor V Leiden mutation, and MTHFR mutation were negative. Antiphospholipid and antinuclear antibodies, anti-dsDNA and anti-ENAs antibodies were negative, serum immunoglobulin levels were normal. High sensitivity flow cytometry confermid the PNH diagnosis as negative markers (CD59 on red cells and CD66b/CD14/CD33/CD24 on polymorphonuclears). Treatment with Eculizumab was strongly recommended but the patient refused the treatment. Patient's clinical condition progressively deteriorated with hyperammonemia and death for multiorgan failure followed after one month. *Conclusions.* PNH is a rare but serious condition. Thrombosis is the leading cause of death in these patients. Hemolysis contributes to TE and TE events increased hemolysis. The tendency toward thrombosis in patients with PNH is multifactorial, involving the absence of GPI-anchored complement inhibitors on the surfaces of circulating platelets, the high levels of intravascular free plasma hemoglobin with the consequent scavenging of NO, fibrinolytic defects, and the proinflammatory effects of C5a. The majority of the mechanisms relate to complement dysfunction and its conequences. Therefore eculizumab, which addresses these mechanisms, resulting in the reduction of thrombosis risk, has now become an important part of the management of this most feared complication. Because thrombosis is the leading cause of death, the impact of eculizumab on thrombosis largely explains the improved survival seen with eculizumab therapy in PNH patients

OUTCOME OF NEWLY DIAGNOSED SYMTOMATIC MULTIPLE MYELOMA (MM) IN PATIENTS AGED 80 YEARS OR MORE: A SINGLE CENTRE EXPERIENCE

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Introduction. MM is a plasma cell neoplasm typical of the elderly, with a median age at diagnosis of 65 years. The increase in median age in western countries has led to an increase in the incidence of this disease; in addition the introduction of novel agents, such as the immuno-modulatory drugs thalidomide and lenalidomide, and the proteasome inhibitor bortezomib, has considerably changed the therapeutic scenario both for young and elderly patients with MM. *Methods*. We report a retrospective analysis of the outcome of 16 very elderly (80 years or more) patients (M/F: 11/5) diagnosed and treated at our Institute from January 2008 to March 2014. Median follow-up was 17 months (range 6-49) after the start of treatment to determine the characteristics of this subset of very elderly patients. *Results*. Median age at diagnosis was 83 years (range 80-89) and PS was <2 in 13 cases (81%). One or two concomitant diseases requiring specific treatments were present in 11 patients (69%), and 3 or more concomitant diseases were present in 5 patients (31%). MM was IgG lambda in 8 patients, IgG k in 4 patients, IgA k in 1 case, IgA lambda in 1 case, and micromolecular in 2 cases. According to the ISS, 8 patients were classified as III stage and 8 patients as II stage. Anemia (median value: 9.6 g/dL) was present at diagnosis in 12 patients (75%) and was the most frequent CRAB feature. Bone lytic lesions were present in 10 patients (62.5%) and zoledronic acid and ibandronic acid were used in 9 and 2 patients respectively. First line therapy was bortezomib (once-weekly administration)/dexamethasone in 9 patients (56%), and melphalan/prednisone +/- thalidomide in 7 patients (44%). According to IMWG response criteria, 3 patients achieved CR, 4 achieved PR, 1 achieved VGPR, and 5 achieved stable disease; 3 patients experienced progressive disease. Hematologic toxicity was infrequent but usually weak/moderate (grades 1 & 2 on the WHO scale). Seven patients received erythropoiesis-stimulating agents. Extrahematologic toxicity was observed in 6 patients (37.5%), and neuropathy was the most common adverse event for treatment. Ten patients (62.5%) had at least one disease progression since diagnosis and were therefore switched to second-line therapy. The median time to first disease progression was 11 months (range 5-28) since start of first-line therapy. Second line therapy was bortezomib (once-weekly administration)/dexamethasone in 3 patients, lenalidomide/dexamethasone in 4 patients, and melphalan/prednisone in 3 patients. 9 patients (56%) are still alive and continue to receive treatment. Six patients died due to disease progression. One patient died due to urinary bladder cancer. *Conclusions*. A study in a larger series of patients is warranted but our experience showed that no upper age limit should be applied for the administration of new drugs with MM; these treatments could be offered to very elderly patients, including those with severe concomitant diseases.

EXTRAMEDULLARY MULTIPLE MYELOMA (EMM) PRESENTING AS SARCOMA

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Introduction. EMM can be defined as a clonal plasmacytic infiltrate at anatomic sites distant from the bone marrow or adjacent soft tissue in a patient with underlying MM. EMM can be present either at the time of initial diagnosis (primary EMM) in approximately 4–16% of MM patients or during further MM disease course (secondary EMM) in approximately 6–20%. Observational studies suggest that EMM is a highly aggressive disease entity, with clinical outcome different from bone marrow restricted MM. In fact the introduction of novel therapeutic regimens has significantly improved the survival of MM patients, but not that of patients with EMM whose survival remains extremely poor. *Methods*. We report the case of a 77-year-old man with no history of trauma admitted to our hospital in June 2013 with a painful mass protruding from the right side of his lower back. The patient reported that the mass had been growing incrementally. Physical examination revealed a solid, nontender mass localized in the upper part of the pelvis. The mass was fixed and not reducible with manual compression. Serum electrophoresis showed an increase of β-globulins; serum immunofixation revealed M-protein (IgA k: 1.19 g/dL) and Bence Jones was positive for k chains. It is known that M-protein can be found in numerous diseases (hematological or other) encountered in clinical practice. Computed tomography of the abdomen confirmed by magnetic resonance imaging showed a voluminous solid formation with a maximum dimension of 9.3 x 12 cm in the area of the right large and medium gluteal muscles extending partially into the small gluteal muscle. Contrast medium highlighted limited areas of colliquative necrosis. The formation caused erosion of the right iliac wing, clearly evident in three-dimensional computer tomography reconstruction of the bone and involving also the iliac muscle. It also caused erosion of the sacrum and spinal muscles on the same side. These aspects led to a

hypothesis of Sarcoma. *Results*. Tru-cut biopsy of lesions was performed and revealed an infiltration of plasma cells (CD20+, CD138+, k chains +). Bone marrow biopsy showed 70% plasma cells (CD20+, CD138+). FISH analysis performed on marrow cells revealed monosomy of chromosome13. A diagnosis of primary EMM was made and the patient started treatment with bortezomib (once-weekly subcutaneus administration) and dexamethasone. After 6 cycles of therapy a reduction of M-protein of 80% and of solid formation of 40% was observed and the patient continues to receive treatment. *Conclusions*. Given that 1) M-protein can be found in numerous conditions encountered in clinical practice, 2) there are no specific radiological features of EMM, and 3) EMM can resemble other neoplasms (including sarcoma), EMM should be included in the differential diagnosis of a mass, especially in patients with monoclonal protein detected in blood, urine, or both.

MULTIPLE MYELOMA IN PATIENT WITH HCV-RELATED DISEASE

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Introduction. Multiple Myeloma is a haematological cancer caused by proliferation of neoplastic monoclonal plasmacells in the bone marrow. The diagnosis of multiple myeloma requires the study of various parameters: clinical (general condition, bone syndrome), biological (study of marrow, study of protein in the blood and urine, hemoglobin level, serum calcium, and creatinine), and radiological (X-ray of the skeleton). In this article we report a case of a 74-year-old women with multiple myeloma and HCV-related desease treated with Bortezomib. *Methods.* In 2008 diagnosis of MGUS. From 1976 Hepatitis HCV-related disease after transfusion. During this period periodic abdominal ultrasound showed steatosis of liver and an increase of transaminases. No need of liver biopsy. In July 2011 it showed evolution in Multiple Myeloma. Stage IIA according to Durie and Salmon Stage, I Stage according to ISS Stage System. Principal characteristics at diagnosis: PLT 73.000/mmc, Hb 9.6 g/dl, WBC 4.100/mmc, Plasmacells 33%, SGOT 120 U/L , SGPT 93 U/L, Creatinine 1 mg/dl, IgG 3060 mg/dl, Calcium 8.4 mg/dl, Gammaglobulin 34.7%, Monoclonal Component 3.1 g/dL, Serum Immunofixation IgG-Lambda, Urine Immunofixation Negative, Scheletal Survey Negative, MRI Negative, FISH Negative, Cytogenetics Negative, HCV-RNA 2.000.000 UI/ml. So the patient was treated with Bortezomib (1,3mg/m^2) and Dexamethasone (40mg/mq) day 1,8,15,22. *Results.* After 9 cycles of therapy, completed in August2012, the patient obtained a VGPR (Very Good Partial Response), with an improvement of liver parameters and of the blood counts. SGOT 36 U/L, SGPT 35 U/L, HCV-RNA 1.890.000 UI/mL, PLT 90.0000/mmc. Currently, the patient is treated with bisphosphonates. *Conclusions.* The efficacy of Velcade in Multiple Myeloma is known to everyone. In this report, we want to show that, although the patient has been affected by HCV-related liver disease for many years, treatment with Bortezomib allows to obtained a good response to Myeloma. Furthermore, the combination with steroids did not worsen the hepatic pathology.

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