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ABSTRACT BOOK

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ABSTRACT BOOK

XV Congress of the Italian Society of Experimental Hematology

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MAIN PROGRAM

IMMUNOGENICITY OF NPM1 AML: POTENTIAL CANDIDATE FOR CTL THERAPY AND IMMUNE CHECKPOINT INHIBITION

J. Greiner[1,2], V. Schneider[2], H. Schrezenmeier[3], M. Wiesneth[3], L. Bullinger[2,4], H. Doehner[2], S. Hofmann[5], M. Goetz[2]

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Abstract: Acute myeloid leukemia (AML) with *mutated nucleophosmin 1* (*NPM1^{mut}*) forms a particular entity in the WHO classification of AML (WHO classification 2017). Overall survival in patients with AML with NPM1^{mut} is more favorable, possibly based on the immunogenicity of the mutated NPM1. Thus, one can hypothesize that AML with NPM1^{mut} might constitute an immunogenic subtype of AML. In this presentation, we demonstrate specific T cell responses against epitopes derived from NPM1 and the mutational region of NPM1, the immunological profile of NPM1^{mut} AML compared to other AML subtypes, PD-L1 expression in NPM1^{mut} AML cells and immune responses against leukemic progenitor cells of NPM1^{mut} AML patients in addition with immune checkpoint inhibitors. We described specific T cell responses against epitopes derived of the mutated region of NPM1^{mut} (Greiner, Ono *et al.* 2012). Additionally, Kuželová and colleagues compared the frequency of human leukocyte antigen allele I (HLA-I) in AML with NPM1^{mut} with NPM1 wildtype AML. They observed that in AML with NPM1^{mut} patients with several HLA-I alleles associated with NPM1 directed immune responses showed a better overall survival (Kuzelova, Brodska *et al.* 2015). In a survival analyses of 25 NPM1^{mut} AML we found that patients that appeared to have specific T-cell responses against one or two of the immunogenic peptides had a better overall survival in comparison to those cases showing no specific immune responses (Greiner, Schneider *et al.* 2013). These findings show that immune responses against the mutated protein of NPM1 might contribute to the favorable prognosis of AML patients carrying NPM1^{mut}. Moreover, our group investigated in microarray analysis whether the enriched leukemic stem cell (LSC) population of AML with NPM1^{mut} shows an expression profile that may make AML with NPM1^{mut} susceptible for immune mechanisms (Schneider, Zhang *et al.* 2014). Immune Checkpoint Inhibition targeting Programmed cell death protein 1 (PD-1)/Programmed cell death 1 ligand 1 (PD-L1) has been proven to be an effective novel immunotherapeutic approach in cancer treatment including the treatment of hematological malignancies. Therefore, we investigated the expression of CD34/CD38/CD274 in 20 *NPM1^{mut}* versus 20 wild-type (*NPM1^{wt}*) AML patient samples via flow cytometry analyses to assess PD-L1 (CD274) expression in leukemic cells, including leukemic progenitor and stem cells (LSC). We also investigated the influence of the anti-PD-1 antibody Nivolumab® on the $_{antigen}$-specific immune responses in ELISpot assays. Additionally, we assessed the effect of Nivolumab in colony forming unit (CFU) immunoassays. Specific T cells for the chosen LAA with and without addition of Nivolumab were generated in mixed lymphocyte peptide cultures (MLPC). Cells from 25 patients were utilized to detect the effect of specifically stimulated CTL against various LAA on the proliferation and formation of colonies of leukemic stem and progenitor cells employing CFU (Colony Forming Unit) assays.

CFU immunoassays showed a significant inhibition of CFU when adding T cells stimulated against various LAA, which were WT1, RHAMM, PRAME and NPM1. In all patient samples, effectors activated against at least one LAA were successful to decrease the colony number significantly. Ten of twelve patients showed and inhibition in the number of colonies in CFU assays of at least one LAA and eight out of ten patients of 2 or 3 LAA. Immune effects increased adding Nivolumab to the CTL for several days before starting CFU immunoassays. In conclusion, AML with *NPM1^{mut}* seems to be an immunological subtype and immunotherapies targeting might be an option to enhance NPM1^{mut} specific T cell responses in order to eradicate persisting MRD following conventional chemotherapy.

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MINIMAL RESIDUAL DISEASE BY MFC IN ACUTE LYMPHOBLASTIC LEUKEMIA IN CHILDREN

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Pediatric acute lymphoblastic leukemia (ALL) prognosis has been improving in the last few decades, with an actual overall survival probability of 80-90%. The improvement in the therapy results has derived from new biologically approaches as well diagnostic approaches, risk classification obtained from biological factors, and development of tailored therapies based on individual therapy response defined on Minimal Residual Disease (MRD) detection. Today MRD evaluation is a golden standard in childhood ALL management and is the strongest predictor of outcome. To be clinically useful, MRD assay should have a sensitivity of at least 1 x 10^{-4}, discriminate blast from normal cells, allow to obtain results promptly, and be consistent when applied in different laboratories. The most reliable methods to detect MRD are Multiparametric Flow Cytometry (MFC) analysis of leukemia-associated immunophenotypes (i.e. aberrant phenotypes expressed in leukemic but not in normal cells) and antigen receptor gene rearrangements, Ig and TCR, as specific sig-

nature of the malignant clone analysed by PCR or NGS. MRD by MFC is applicable to almost all the patients and provides prognostic information that overrides classical risk factors. MFC is an ideal MRD assay, with a high sensitivity and specificity, it allows to obtain results promptly, results are consistent when applied in different laboratories and cheaper than PCR MRD. MFC-MRD is defined as positive when a cluster of cellular events with homogeneous immunophenotype can be detected. To obtain a sensitivity of 1 x 10-4 cells, the acquisition of at least 1 x 105 nucleated cells is needed but 1x106 is considered better. To analyze MRD by MFC, the primary gate should be set as an immunological gate: CD7 versus side scatter (SS) in T-ALL, and CD19 versus SS in B-cell precursor ALL, respectively. In T-ALL, MFC MRD on bone marrow and peripheral samples aims to detect immature cells, usually confined to the thymus. In B-cell precursor ALL, blast cells can be distinguished from normal B-cell progenitor in bone marrow by aberrant marker expression patterns. The identification of new markers to be used in MRD by MFC in ALL was traditionally based by comparing the immunophenotype of normal and leukemic cells or, more recently comparing the gene expression profile of normal and leukemic CD19+ CD10+ cells, searching for differentially expressed genes (e.g. CD58). To notice, antigen expression can be modulated during the first therapeutic phase (steroid phase) with changes in immunophenotypic patterns, especially at Day 15: this bias is generally transient (at least for CD10, CD20 and CD34) and reverts to initial patterns after stopping steroid therapy. In particular, an AIEOP study defined 3 risk groups according to MFC-MRD on bone marrow samples at Day 15: standard (< 0.1%), intermediate (0.1% to <10%), and high (≥10%), associated with a 5-year cumulative incidence of relapse of 7.5%, 17.5%, and 47.2% respectively. The strength of these data justified, in the subsequent AIEOP-BFM-ALL trial for treatment, the use of MFC at day 15 to define the final risk group in those patients without informative PCR-MRD marker, to identify a high risk group of patients (blast cells ≥10%) for therapy intensification and a low risk group of patients (< 0.1%) randomized for therapy reduction. The availability of measurement of MRD at early time point with a reduced-cost method is of clinical relevance: the ALLIC-BFM 2002 trial designed for countries with inadequate resources for PCR-based MRD monitoring combines morphologic results of PB at day 8 and BM by MFC at day 15 and day 33. Moreover, based also on AIEOP-BFM-ALL 2009 results the day 15 MFC-MRD is one relevant prognostic marker in the new AIEOP BFM 2017 ALL protocol. In conclusion, MFC is an accurate and prompt technique to detect response to therapy and impending relapse, even when "isolated" (extra-medullary relapse). Many efforts were made to standardize MFC methods for MRD assessment by different collaborative groups. Among different multicentric studies, AIEOP-BFM laboratories worked together to harmonize each step of this technique, the whole process from sample preparation to data interpretation.

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Coustan-Smith E, Song G, Clark C, et al. New markers for minimal residual disease

CELLULAR HETEROGENEITY AND CLONAL EVOLUTION IN XENOGRAFT MODELS OF ACUTE MYELOID LEUKEMIA (AML)

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Acute myeloid leukemia (AML) is one of the most frequent hematological malignancies in adults, and still represents a disease with an unmet medical need, with 50-60% of patients relapsing within 3 years after diagnosis (Dohner, Weisdorf *et al.* 2015). AMLs are characterized by a high degree of intra-tumor heterogeneity, both at the biological and the genetic level, which is critical for tumor maintenance and response to treatments. Biologically, AMLs are organized hierarchically, with rare stem-like cells (leukemia stem cells, LSCs) endowed with the unique properties of self-renewal and differentiation (Bonnet and Dick 1997). Genetically, AMLs harbor patient-specific combinations of different driver mutations, which are organized within individual cases in sub-clones with distinct growth properties (Grove and Vassiliou, 2014; Klco, Spencer et al. 2014; Paguirigan, Smith et al. 2015; Papaemmanuil, Gerstung et al. 2016). We hypothesized that tumor maintenance and relapse in AMLs are driven by the selective expansion of quiescent subclones within the LSC population, which serve as the genomic and functional reservoir of the tumor. The experimental strategy we employed to test this hypothesis was based on the xenotransplantation of human leukemias, the implementation of an *in vivo* clonal tracking approach, the functional isolation of leukemic subpopulations with diverse proliferation histories and whole-exome sequencing (WES) of bulk and isolated leukemic subpopulations. We identified two functional LSC classes, quiescent and cycling, that are in equilibrium in the tumor and largely share the same clonal architecture. We further observed that genetic leukemic clones appear to consist of a high number of individual LSCs, the majority of which exhaust upon serial transplantation. Finally, by genetic analyses of isolated leukemic subsets, we were able to detect a specific enrichment for rare mutations in the quiescent compartment of patient xenografts, which can be selected under the environmental pressure of chemotherapy. Our data indicate that tumor evolution is sustained by the quiescent LSC pool and suggest that long-term survival and propagation of individual LSC clones relies on their ability to preserve a quiescent compartment. Upon selective expansion of quiescent LSCs, minor genetic sub-clones carrying mutations associated with tumor aggressiveness and chemotherapy-resistance can emerge, suggesting a mechanism for the development of refractory relapse tumors.

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AGEING, INFLAMMATION AND METABOLIC STRESS UPSET THE ENERGY CHARGE OF THE BODY: HOW DOES PURINERGIC SIGNALLING IMPACT ON THIS DELICATE BALANCE

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Ageing is often paralleled by an inherent inability of cells to sustain an efficient energy metabolism, whether glycolytic or oxidative. This translates into morphological changes of the mitochondrial network that is "thinner", fragmented and sparser.[1] At the same time, ageing is characterized by an "inflammatory" phenotype that accelerates and possibly promotes the overall aging process. It is nowadays textbook knowledge that inflammatory sites have a high concentration of extracellular nucleotides, mainly ATP, the prototypical DAMP (damage-associated molecular pattern).[2] Extracellular ATP acts at plasma membrane receptors named P2 purinergic receptors, that function to all means and purposes as "sensors" of the extracellular ATP concentration, and therefore of the "energy charge" of the extracellular environment.[3] In the P2 receptor family, the subtype most closely associated to inflammation is the P2X7 receptor (P2X7R) subtype, an ATP-gated ion channel with peculiar permeability properties. The P2X7R has a striking effect on cellular energy metabolism because is supports both oxidative and glycolytic metabolism, but at the same time, when overactivated, the P2X7R precipitates a mitochondrial catastrophe leading to cell death.[4] Since the "energy charge" of the extracellular environment is a reflection of the intracellular ATP content, the P2X7R is at the same time sensor of the extracellular ATP concentration and a factor that, by modulating the intarcellular ATP level, dictates the level of extracellular ATP, and therefore the level of its own agonist. The increased concentration of extracellular ATP at inflammatory sites disrupts the energy homeostatic function of the P2X7R and impairs the overall cellular fitness.[5] Not surprisingly, P2X7R expression negatively correlates with ageing in the western world (but not in developing countries), and P2X7R activity accelerates progression of ageing-associated diseases such as Type 2 Diabetes and atherosclerosis. Pharmacological blockade of the P2X7R may offer hopes to alleviate ageing-associated diseases.

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GENOMICS, AGING AND MOLECULAR THERAPIES

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Aging of the hematopoietic system is associated with diminished regenerative potential, as a result of reduced hematopoietic stem cell long-term self-renewal capacity, skewing of hematopoietic cell differentiation with increased myeloid lineage output and markedly decreased output of cells of the lymphoid and erythroid lineages, changes in metabolism and signaling pathways, accumulation of genome aberrations, changes to the epigenome and defects in the stem cell niches. These changes are in turn associated with an increased prevalence of anemia and other peripheral cytopenias, and an increased risk of developing hematologic cancers, including myeloid neoplasms.[1]

Recently, large studies provided consistent evidence of age-related hematopoietic clones in up to 10% of persons 70 to 79 years of age, and 20% of persons 90 years of age or older. Detectable clonal expansions most frequently involved somatic mutations in three genes implicated in epigenetic regulation, *DNMT3A*, *TET2*, and *ASXL1*. The vast majority of subjects carrying detectable mutations had only one mutation, supporting the hypothesis that these persons had clones harboring only an initiating lesion. In addition, the median variant allele fraction for the identified mutations up to 20%, suggesting that the variants are present in only a subset of blood cells.[2,3] The terms of Age-Related Clonal Hematopoiesis (ARCH) or Clonal Hematopoiesis of Indeterminate Potential (CHIP) have been introduced to define the condition characterized by the presence of a hematopoietic clone in the absence of diagnostic criteria for neoplasm.

Persons with clonal hematopoiesis had a risk of developing hematologic cancers elevated by a factor up to 13 than subjects without any detectable putative somatic mutations. Several lines of evidence suggest that ARCH may represent an initial stage of malignant transformation of a hematopoietic cell. In fact, driver genes are consistently mutated in either clonal hematopoiesis and hematologic malignancies. In addition, the number of driver mutation per subject, as well as variant allele frequency, are progressively increasing from clonal hematopoiesis to malignancy, supporting the hypothesis that age-related hematopoietic clones have the potential to evolve into hematologic neoplasm through the acquisition of additional cooperating mutations.[4] Accordingly, studies conducted in registries of patients with solid tumor recently showed that the detection of ARCH at the time of exposure to chemo-radiotherapy was associated with a significant increase in the risk of developing a therapy-related myeloid neoplasm.[5,6] Recent experimental and clinical evidence suggested that ARCH is also associated with a significantly higher risk of cardiovascular mortality, as a consequence of accelerated atherosclerosis. Mutations in *DNMT3A*, *TET2*, *ASXL1*, and *JAK2* were proven to be individually associated with coronary heart disease. In addition, hypercholesterolemia-prone mice, engrafted with *Tet2* knockout bone marrow cells, developed more severe atherosclerotic lesions. Preliminary *in vitro* and *in vivo* data are suggesting that these hematopoietic clones showed elevated expression of several chemokine and cytokine genes, resulting in increased inflammation.[7,8]

Finally, the accessibility of massive genome sequencing technologies and the prospective availability of safe and effective targeted therapeutic approaches interfering with selected mutations driving age-related hematopoietic clones, if confirmed in *ad hoc* clinical studies, are paving the way strategies of screening and early therapeutic interventions in individuals with clonal hematopoiesis.[9,10]

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MINIMAL RESIDUAL DISEASE (MRD) IN ACUTE LYMPHOBLASTIC LEUKEMIA: THE GIMEME/NILG ESPERIENCE

A. Vitale[1], O. Spinelli[2], F. Paoloni[3], I. Della Starza[1], A. Santoro[4], L. Elia[1], M. Tosi[2], V. Apicella[1], D. Salemi[4], M. Paris[2], M. Cavalli[1], M. Vignetti[3], A. Guarini[5], F. Fabbiano[3], A. Rambaldi[2,6], R. Foa[1], R. Bassan[7] on behalf of GIMEMA study group (Table 1)

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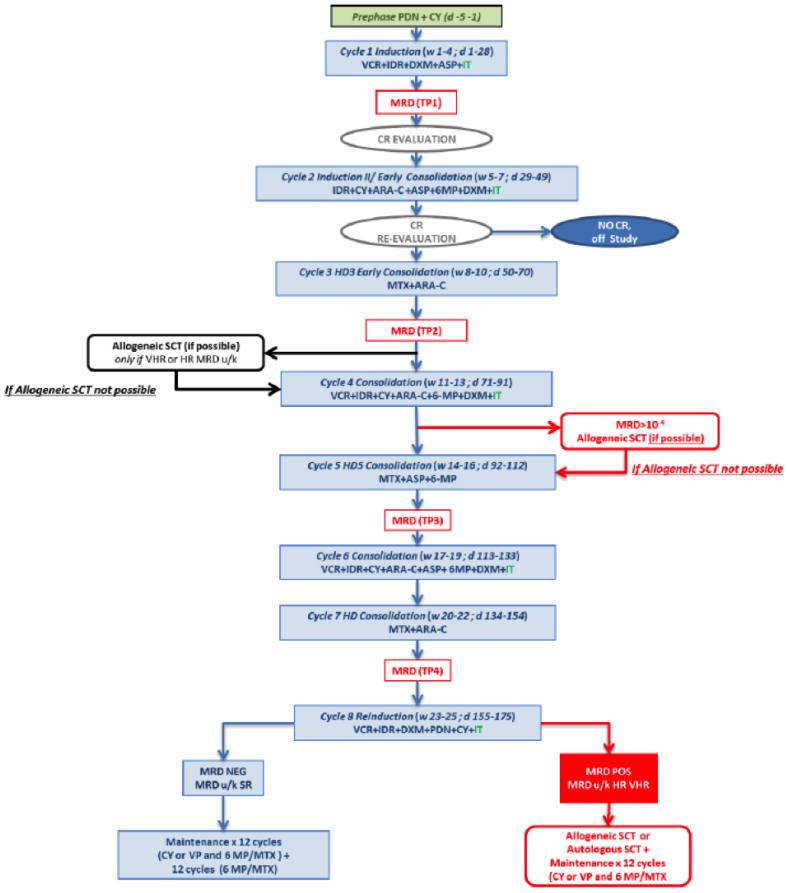
Acute lymphoblastic leukemia (ALL) is a rare disease in adults. Until very recently, complete remission (CR) rates of 85-90% and disease-free survival (DFS) rates of 40-50% at 3-5 years have been reported, for an average overall survival (OS) rate of 35-45%.[1,2] Several prognostic variables can affect outcome and modify these data substantially. One crucial question is whether prognostic factors can be used to modify therapeutic choices in individual patients. This requires the recognition of reliable risk factors and the availability of different treatments that are more or less indicated for different risk classes.

Available data in adult ALL from retrospective/prospective clinical series have underlined the role of minimal residual disease (MRD) as a major prognostic indicator capable of allocating patients to MRD-oriented therapies.[3] According to the recommendations of the EWALL group,[4] MRD assessment is mandatory for risk stratification, risk-oriented therapy with or without stem cell transplant (SCT) and disease response monitoring for alternative treatments in MRD-positive patients.

With the above reviewed background, the GIMEMA group has recently completed a phase II trial that adopted a flexible risk/MRD-ori-



Patients: 203 (including LL)
Inclusion criteria: 18-65 years
Based on:

- Derived from the NILG 10/07 trial.
- An updated induction/consolidation regimen that includes pediatric-derived elements, such as Peg-ASP and lineage-targeted MTX infusions: Pediatric-inspired regimen
- A case-specific risk classification with early MRD evaluation (all patients): MRD driven strategy
- A risk-oriented consolidation with allogeneic SCT (or autologous SCT when the former is not possible) as preferred therapeutic option for MRD-positive SR/HR patients (the risk being primarily defined by MRD analysis) and all VHR cases, and consolidation/maintenance for MRD-negative SR/HR patients; an additional mediastinal irradiation for LL patients with residual CT/PET positivity.

Figure 1. GIMEMA LAL 1913.

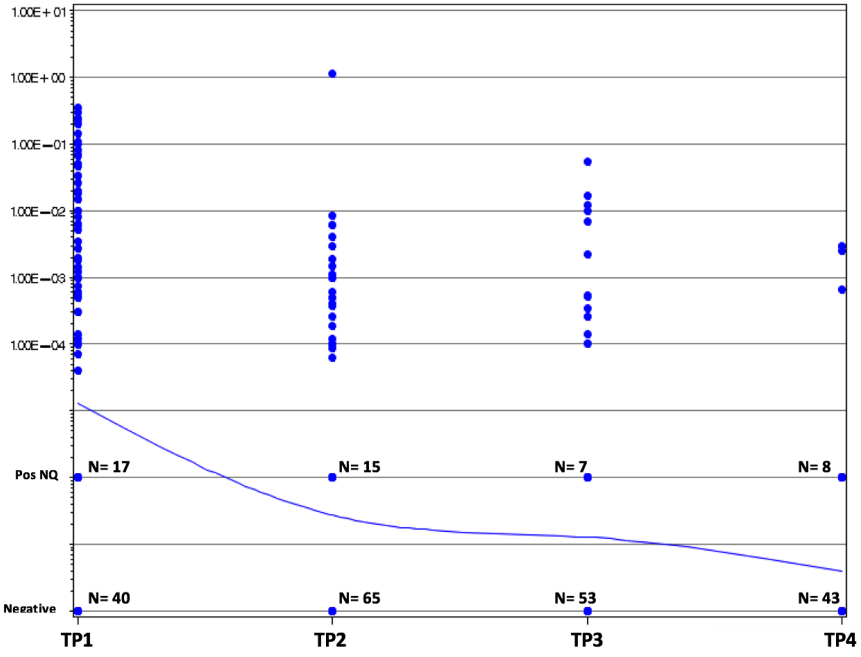


Figure 2. The MRD levels at different time points.

Table 1. Participating Centers with at least four enrolled patients.

Clinical Center	Enrolled patients
012 Milano Ospedale Niguarda Ca Granda - SC Ematologia	9
037 Torino Dipartimento di Oncologia ed Ematologia S.C. Ematologia 2 A.O. Citta' della Salute e della Scienza di Torino San Giovanni Battista	9
042 Pavia S.C. Ematologia - Fondazione IRCCS Policlinico S. Matteo	9
055 Brescia Spedali Civili - Brescia - Azienda Ospedaliera - U.O. Ematologia	9
006 Bologna Istituto di Ematologia Lorenzo e A. Seragnoli - Universita' degli Studi di Bologna - Policlinico S. Orsola - Malpighi	8
008 Catania Universita' di Catania - Cattedra di Ematologia - Ospedale Ferrarotto	8
028 Roma Universita' degli Studi Sapienza - Dip Biotecnologie Cellulari ed Ematologia - Divisione di Ematologia	8
086 Mestre U.O. di Ematologia- Ospedale dell'Angelo - Mestre	7
030 Roma U.O.C. Ematologia - Ospedale S.Eugenio	6
034 Udine Clinica Ematologica-Centro Trapianti e Terapie cellulari Azienda Ospedaliero-Universitaria, Udine	6
045 Milano Fondazione IRCCS Ca Granda Ospedale Maggiore Policlinico UOC Oncoematologia- Padiglione Marcora	6
005 Bergamo Azienda Ospedaliera - Papa Giovanni XXIII	5
041 Bolzano Comprensorio Sanitario di Bolzano - Azienda Sanitaria dell'Alto Adige - Ematologia e Centro TMO - Ospedale S.Maurizio	5
043 Verona Universita' degli Studi di Verona - A. O. - Istituti Ospitalieri di Verona- Div. di Ematologia - Policlinico G.B. Rossi	5
048 Cuneo S.C. Ematologia ASO S. Croce e Carle	5
067 Siena U.O.C. Ematologia e Trapianti - A.O. Senese - Policlinico Le Scotte	5
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ented therapeutic approach derived from the NILG 10/07 study entitled: "National Treatment Program for Philadelphia Chromosome-Negative Adult Acute Lymphoblastic Leukemia with Pegylated Asparaginase Added to a Lineage-Targeted Risk-and Minimal Residual Disease-Oriented Strategy. GIMEMA LAL 1913" (ClinicalTrials.gov Id: NCT02067143) (Figure 1). Between December 2014 and September 2016, 203 patients were enrolled in the GIMEMA LAL 1913 protocol. For the current analysis, we considered only the patients with ALL and not those with lymphoblastic lymphoma. Thus, 178 Ph-ALL were evaluated for clinico-biologic features at presentation of the disease: 78 were females and 100 males; the median age was 39.84 years (range 18.18–65.11) and the median WBC count was $6.01x10^9$/L (range 0.12–367.30). Flow cytometry analysis showed that, as expected, there was an overall prevalence of B-lineage ALL (75.28%), while T-ALL was much less frequent (24.72%). Twenty-three patients were molecular transcript-positive and 155 resulted negative. These latter cases were studied for clonal Ig/TCR rearrangements identification and quantitative MRD assay development. This procedure was successful in 86% of studied patients with a similar success rate in the three reference laboratories. The MRD assays reached at least a sensitivity of 10^{-4} (i.e. one leukemic cell out of 10.000 normal cells) according to the EuroMRD Guidelines.[5]

Of the 178 patients, only 1 did not start chemotherapy due to a concomitant infection. After the first cycle of induction (TP1 evaluation), 147 (84.48%) patients showed a complete hematological remission (CHR), 15 resulted resistant (8.62%), 3 were not evaluable for bone marrow hypo-cellularity and in 3 patients data are missing. There were 9 deaths (5.17%) in induction. After the second cycle of induction (C2), 11 additional patients obtained a CHR (9 among the resistant cases and 2 among the not evaluable); as per protocol indication, these cases could be included in the final assessment of CHR patients (n=158, 90.8%). The TP1 for CHR evaluation was also the first time point for MRD measurement followed by a subsequent evaluation after cycle 3 (HD3 early consolidation, TP2), 5 (HD5 consolidation, TP3) and 7 (HD7 consolidation, TP4). The MRD levels at the different time points (Figure 2) allowed to identify patients with a higher risk of relapse (MRD $\geq 10^{-4}$ at TP2 or later) who, therefore, became eligible to treatment intensification (SCT). Patients negative or with low MRD levels ($<10^{-4}$) could proceed with chemotherapy cycles and maintenance as final consolidation. The median follow-up of the study is 22.7 months (range: 2.0-42.5). The median OS has not yet been reached, with an OS at 12 months of 81.0% (CI 95%: 75-87.4). In addition, the median DFS has not yet been reached and the DFS at 12 months is 78.3% (CI 95%: 71.7- 85.6).

The results of this study confirm that a MRD-based treatment protocol for adult ALL is feasible on a national basis through a centralized handling framework and MRD measurement by specialized laboratories performing adequate quality control assessments within European Working groups. MRD-based therapy modulation allowed obtaining very promising OS and DFS both in B- and T-lineage ALL. Nevertheless, a proportion of patients still relapse, especially those with high MRD levels, emphasizing the need of additional experimental treatment strategies aimed at further reducing leukemia burden. The current national MRD-driven protocol – GIMEMA LAL 2317 - indeed incorporates the bi-specific monoclonal antibody blinatumomab in the chemotherapy backbone ~~for Ph- ALL~~ (Id: NCT 03367299).

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MINIMAL RESIDUAL DISEASE IN NON HODGKIN'S LYMPHOMAS

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In the era of target therapy, the minimal residual disease (MRD) represents a debated topic, because its prognostic role is emerging in different hematological malignancies, and new sensitive molecular techniques are now available. The "MRD" is a wide definition, including all imaging, flow cytometric, and molecular tests allowing to detect disease at the level where the "conventional" tools don't arrive. Today, the availability of new flow cytometers, of digital PCR (D-PCR), and of the deep sequencing (NGS) methods allowed the significant technical improvement recently observed also in the MRD field. Differently from acute leukemias, in non Hodgkin's lymphomas MRD is more frequently assessed by molecular instead of flow cytometric assays. The first step is the identification of a molecular marker (better if patient-specific) that will be followed after treatment and during the follow-up. In the follicular lymphoma, half of patients carry the BCL2/JH rearrangement, in mantle cell lymphoma about 40% presents the BCL1/JH fusion gene, and the rearrangement of the immunoglobulins heavy chains (IgH) or of T cell receptors (TCR) can be used for all B- and T-cell lymphoproliferative disorders. Today, the real-time quantitative PCR remains the "gold standard" technique, with a sensitivity of 10^{-5}; in particular, the "patient oligonucleotide allele-specific PCR (ASO-PCR)" is the most frequently used.[1] But this method is laborious and time-consuming, requiring the sequence and design of specific primers/probes couples necessary for performing the quantitative PCR. Moreover, this method allows detection of the only pathological clone identified at diagnosis, but it has now well known the existence of the "clonal shift" phenomenon (a clone not identified at diagnosis can survive to treatment and to be the cause of relapse), and it is evident that the use of a patient-specific primers/probe combination is not able to identify eventual different clones. This problem can be avoided by NGS, that is able to detect all clones, including the emerging ones, in a "unsupervised" way. One of the further objects of discussion is what is the right compartment where MRD has to be assessed; traditionally, it is the bone marrow, where the sensitivity is one log higher than in peripheral blood. Nevertheless, more than one quarter of relapses are extra-medullar; the cell-free DNA (cf-DNA), circulating in the plasma, has been reported to be more prognostic than bone marrow or peripheral blood, either in DLBCL[2] or in Waldenstrom macroglobulinemia.[3] About the prognostic role of MRD, the majority of information derived from trials on follicular and mantle-cell lymphoma: the history of MRD in FL started with the demonstration that rituximab significantly reduced the molecular tumor burden when administered after the CHOP, with patients achieving MRD negativity showing a longer event-free survival.[4]. Analogously, in the autologous transplantation, patients MRD-negative after transplant and re-infused with molecularly-free harvests remained relapse-free and alive in a higher percentage than MRD-positive cases.[5,6] Subsequently, when yttrium-ibritumomab tiuxetan as consolidation was introduced, it was evident that radio-immunotherapy was able to eradicate MRD in more than 70% of patients, with a significant favorable impact on progression-free survival.[7] Then, two fundamental studies by the Fondazione Italiana Linfomi (FIL) showed that, after R-CHOP, R-CVP, R-FM, and R-FND, the MRD status did significantly impact on outcome: patients in partial remission but MRD-negative showed longer PFS than those in complete remission but still MRD-positive[8,9](see Figure 1). More recently, the appearance of the new anti-CD20 monoclonal antibody, obinutuzumab, prompted researchers to evaluate its value also in terms of molecular response. In the Gallium trial, obinutuzumab resulted more powerful than rituximab, offering 92.5% of MRD-negativity when combined with bendamustine, 91.3% with CHOP, and 91.4% with CVP.[10] In Italy is now *in fieri* the FIL FOLL12 study (trial.gov identifier number: NCT02063685), where the experimental arm is based on the PET and MRD results, with a de-intensified treatment for MRD-/PET-negative cases, consolidation with radio-immunotherapy for all PET-positive cases, or pre-emptive therapy for PET-negative/MRD-positive cases. The

aim of this phase-3 trial is the possibility of avoiding the maintenance with rituximab (with reduction of costs and toxicities) for cases with minimal risk of relapse, and of intensifying treatment for cases with low probability of remaining disease-free. We hope that the results of this study could induce to definitively adopt MRD as decisional tool in the clinical practice.

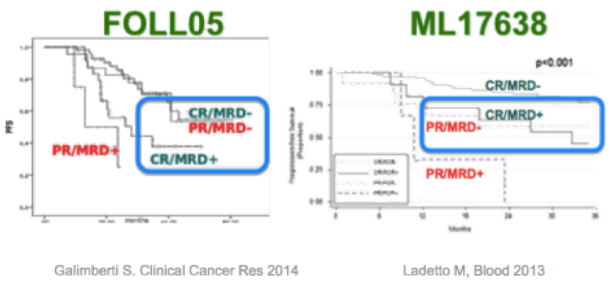

Figure 1.The impact of MRD on the clinical outcome in the Italian experience.

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B-LYMPHOID TRANSCRIPTION FACTORS AND CELLULAR METABOLISM IN ACUTE LYMPHOBLASTIC LEUKEMIA

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Several metabolic changes are required for cell growth, and cancer cells show metabolic alterations driven by a combination of genetic lesions and non-genetic factors, such as changes in tumor microenvironment. A better understanding of this heterogeneity may enable the development and optimization of therapeutic strategies that target tumor metabolism.

Transcription factors critical for B cell development (*i.e.* IKZF1, EBF1 and PAX5) are frequently affected by mutations, deletions and translocations in B-cell precursor acute lymphoblastic leukemia (BCP-ALL), and some of them (*i.e.* IKZF1 and PAX5) have been found to carry germline mutations in rare cases with predisposition to leukemia. However, the pathogenetic significance of these aberrations has still to be demonstrated.

Interestingly, recent data indicates that PAX5 and IKZF1 function as metabolic gatekeepers, by restricting glucose uptake, and therefore glycolysis and oxidative phosphorylation, thus limiting energy supply to levels that impair transformation of pre-B cells. Indeed, compared to myeloid leukemia cells which show high energy reserve, BCP-ALL cells are characterized by a state of chronic energy deficit. In fact, pre-B ALL cells divide faster, are significantly smaller and carry less mitochondria than myeloid leukemia cells, with also lower ATP reserves. This energy deficit triggers the activation of the energy-stress sensor LKB1-AMPK pathway.

The metabolic gatekeeper function may be responsible for the constraint in a latent state of pre-leukemic clones carrying potentially oncogenic lesions. On the other hand, PAX5 and IKZF1 may also modulate sensitivity to glucocorticoids through positive regulation of *NR3C1* expression and suppression of AKT activity in pre-B ALL.

Interestingly on this line, fasting selectively inhibits the development of B-lineage ALL, while obesity and hyperglycemia are associated with poor outcome in patients with pre-B ALL.

This unpredicted function of the B-lymphoid transcriptional program as a metabolic barrier against malignant transformation of BCP cells will be discussed.

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THE DUAL METABOLISM-CONTROLLED STEM CELL NICHE: A SIMPLE MODEL FOR CHRONIC MYELOID LEUKAEMIA RESISTANCE TO THERAPY

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We previously showed that the incubation of Chronic Myeloid Leukaemia (CML) cells in atmosphere at very low oxygen tension drives rapid glucose consumption, as expected, and the adaptation of a cell subset to the shortage of both oxygen and glucose. Under this condition, the oncogenic driver of CML, the BCR/Abl protein, is suppressed, while *BCR/abl* transcript is maintained at least in part.[1-3] Therefore, cells adapted to energy shortage remain genetically leukaemic, so that they are capable to generate, once transferred into a growth-permissive environ-

ment, a BCR/Abl$_{protein}$-expressing progeny that rescues BCR/Abl-dependent clonal expansion. We also found that stem cell potential is maintained under severe energy shortage, implying that BCR/Abl$_{protein}$-negative leukaemic stem cells (LSC) are refractory to the inhibitors of tyrosine kinase activity (TKi) of BCR/Abl currently used for CML therapy, due to the lack of molecular target of TKi.[1,2] Although extremely effective in inducing CML remission, TKi are unable to cure disease, *i.e.* to prevent CML relapse upon discontinuation of therapy, due to the persistence of TKi-resistant Minimal Residual Disease (MRD).[4,5] We envisioned on these bases a simple model predicting that, within stem cell niches of bone marrow where LSC most likely reside, BCR/Abl$_{protein}$ is suppressed in areas where energy shortage is more severe.[6] There, an LSC subset would survive and cycle independently of BCR/Abl signaling, *i.e.* exhibiting loss of oncogene addiction.[7] This LSC subset would sustain MRD (Figure 1). An unconventional strategy to suppress LSC of CML via the targeting of their metabolic adaptation with inhibitors of hypoxia-inducible factors (HIF) has been successfully tested in our laboratory.[8] HIFalpha stabilization, however, occurring at oxygen concentrations as high as 3%,[9] is a necessary but not sufficient condition for the metabolic adaptation of LSC to the very low oxygen tensions (0.1-0.2% O_2) and energy levels of our niche model. Current work is focused on the deepening of the upstream regulation of transition between the BCR/Abl$_{protein}$-negative and -positive LSC phenotypes, as balanced within the most probably "dual" metabolic nature of LSC niche,[6] and to determine whether a "metabolic symbiosis" exists between the two areas of the niche (Figure 1). In this context, we needed to take into account that glucose is not the only fuel of cell metabolism and that lactate and glutamine are likely to condition CML cell adaptation to the stem cell niche environment. The role of glutamine was explored in the experiments summarized here. CML cell lines were chosen for basic biochemical studies to obtain the maximal repeatability of results. K562 and KCL22 cells were plated in liquid cultures (LC) supplemented or not at time zero with glutamine (2 mM) and/or the glycolysis inhibitor 2-deoxy-glucose (2-DG; 1 mM) and incubated in atmosphere at 0.1% O_2 for 4 to 7 days (LC1). At the end of LC1, proteins or mRNA were detected in cell lysates, pH and metabolite concentrations determined in culture medium and the overall stem/progenitor cell potential estimated. To this purpose, LC1 cells were replated into drug-free cultures in standard medium incubated at 21% O_2 (LC2), and the capacity of LC1 cells to repopulate LC2 measured (Culture Repopulation Ability - CRA assay).

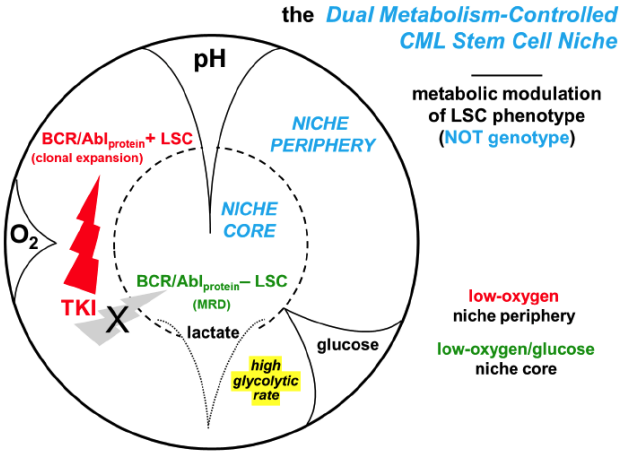

Figure 1.

In culture medium of glutamine-containing cultures incubated at 0.1% O_2, glucose was time-dependently consumed, lactate released and pH lowered. In the absence of glutamine, lactate production and medium acidification were reduced, while BCR/Abl$_{protein}$ expression and BCR/Abl-dependent signaling (Crkl phosphorylation) were maintained. Furthermore, in the presence of glutamine, the expression of glucose transporters on cell surface was reduced, thus accelerating the exhaustion of intracellular glucose. A cause/effect relationship between this exhaustion and BCR/Abl$_{protein}$ suppression emerged from the finding that the addition of 2-DG to glutamine-containing cultures mimics the effects of the absence of glutamine. This indicates that glutamine is crucial to drive glucose catabolism and consequently BCR/Abl$_{protein}$ suppression in low oxygen. When stem/progenitor cell potential was evaluated, LC1 cells incubated in the presence of glutamine repopulated LC2 of CRA assays after a long lag phase, a repopulation kinetics typical of BCR/Abl$_{protein}$-negative LSC, related to the time necessary for BCR/Abl$_{protein}$ re-expression, which then sustains clonal expansion.2 On the contrary, cells from glutamine-free LC1 repopulated LC2 immediately, a kinetics typical of LSC where BCR/Abl signaling is maintained. Thus, glutamine emerged as a crucial metabolite driving the selection of the BCR/Abl$_{protein}$–negative LSC phenotype refractory to TKi as well as LSC adaptation to the microenvironmental conditions of stem cell niches where TKi-resistant MRD is believed to be selectively maintained (Figure 2).

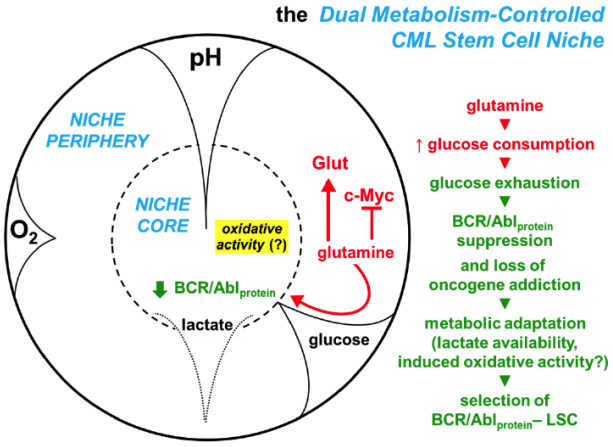

Figure 2.

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METABOLIC REPROGRAMMING IN MYELOMA CELLS AND BONE MARROW MICROENVIRONMENT: ROLE OF GLUTAMINE

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Metabolic adaptation has been identified as major feature of cancer cells[1] including that of multiple myeloma (MM). MM cells are characterized by the tight relationship with the bone marrow (BM) microenvironment that exerts a critical role in the regulation of MM cell grow and survival. In turn MM cells induce a significant alteration of the microenvironment leading to immunosuppression, increased angiogenesis and bone destruction.[2] Several data indicate that cancer metabolism satisfies the high demand for nutrients but also has a possible impact on the tumor microenvironment.[1]

Recently, we have demonstrated that MM cells are typically "glutamine addicted" strictly depending for their growth to the glutamine (Gln) availability[3], as also described for many others tumors.[4] Gln addiction in MM cells is due to the lack of the expression of Glutamine Synthetase (GS) and the overexpression of Gln transporter ASCT2 that is involved in the increased Gln up-take that occurs in MM cells.[3] Moreover we demonstrated that in the BM microenvironment of MM patients Gln is lowered, while glutamate (Glu) and ammonium were increased as compared to patients with premalignant monoclonal gammopathies.[3,5] Consequently we hypothesize that the peculiar low-Gln, high-Glu microenvironment may have a significant impact on the relationship between MM cells and their microenvironment, particularly in the development of bone disease and the progression of MM. Firstly, we demonstrated that stromal, osteoblastic and preosteocytic cells are sensitive to Gln depletion with an higher dependence for osteoblasts and preosteocytes. Indeed, in Gln deprivation, all three cell lines induced the expression of GS suggesting that these cells exploit GS to counteract the shortage of Gln. Secondly, we found that extracellular Gln and Glu influence osteoblast and osteoclast formation. In particular, the treatment of stromal cells with different concentration of Gln and Glu under osteogenic medium, showed that low-Gln, high-Glu levels, similar to those found in BM MM plasma, inhibited osteoblast differentiation. Moreover, the same condition seems to have a positive effect on osteoclast differentiation and formation whereas Glu-free condition has a dramatic effect on osteoclastogenesis.

We also studied whether GS induced-overexpression in MM cells may affect osteoblast and osteoclast differentiation and activity leading

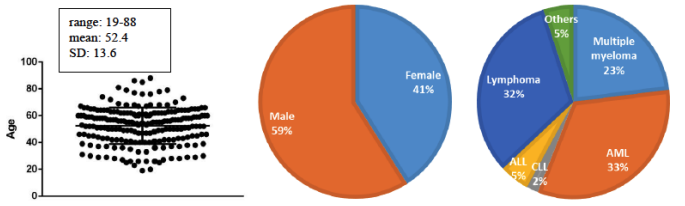

Figure 1. Demographic characteristics of the patients.

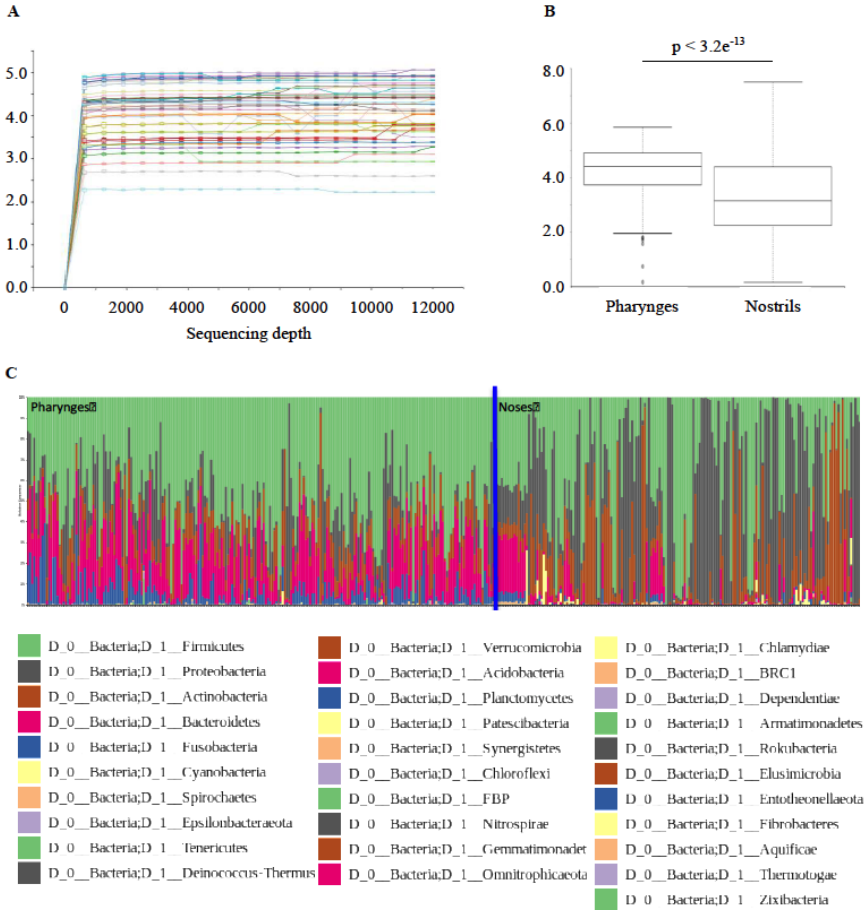

Figure 2. A) Multiple Rarefaction curves calculated from each sample based on Shannon diversity index. B) Boxplot of sample richness between pharynges and nostrils evaluated by Shannon diversity index. Statistical analysis was performed by Kruskal-Wallis (pairwise) test and the corresponding p-value, explicitly shown, indicates a significant difference in the between the groups. C) The histograms show the taxonomic composition at phylum level for each sequenced sample emphasizing the difference between pharynges (left) and noses (right) divided by a blue line.

to bone destruction. BM levels of Gln and its metabolites were correlated with the presence or the absence of bone disease in MM patients. Results are consistent with the hypothesis that low GS expression and Gln addiction of MM cells are functional to osteoblast impairment in the BM microenvironment and the development of osteolytic bone destruction in MM patients.

The study of metabolic reprogramming of MM cells and of their microenvironment allows to clarified the pathophysiological mechanisms involved in the alteration of the MM microenvironment leading to the identification of possible new therapeutic targets in MM patients.[6] New drugs targeting the Gln metabolism as CB-839 are tested in combination with proteasome inhibitor as bortezomib and carfilzomib[3,7] showing synergistic effects and are under clinical investigation.

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THE MICROBIAL LANDSCAPE OF HEMATOLOGICAL PATIENTS AT RISK FOR ASPERGILLOSIS

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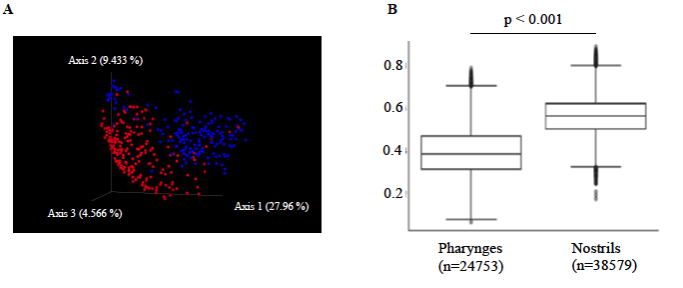

Figure 3. A) PcoA result (Nostrils-blue and Pharynges-red) on the first three principal coordinates evaluated by means of PcoA. B) Boxplot of beta diversities distances of each pharynx sample to all other pharynges and to all nostrils estimated with the weighted unifrac metric. The n index shown in each boxplot is the number of evaluated beta diversities. The p-value obtained with the Permanova pairwise test between groups is also reported.

We have conducted a multicenter, prospective observational study on: "SURVEY OF NASAL MICROBIAL INTERACTION WITH FUNGI" (SNIF Acronym) in order to explore metagenomics for deciphering the contribution of the microbiota to fungal infectivity in the lung and metabolomics for capturing the dialogue between the mammalian host and lung microbiota.[1,2]. By correlating changes in metabolite profiles with microbiota metagenomic composition, the aim is to define functional nodes by which certain bacteria species contribute to or subvert host-fungal symbiosis and mucosal homeostasis in the lung.[3,4] A total of 1472 samples from 212 caucasian patients with different hematological diseases recruited from November 2015 to November 2017 from 7 Italian participating centers were analyzed. From each patient, up to six nasal and oropharyngeal swabs were collected over a six-month period. Figure 1 shows the demographic characteristics of the patients, in terms of age, gender and hematological diseases.

For metagenomics, a 16S target sequencing has been performed on an Illumina platform (300 bp read on Miseq V3) by obtaining 23 million read pairs. All sequenced samples have been processed with the next-generation microbiome bioinformatics platform "QIIME 2™ 2018.4" in order to evaluate both compositional and diversity (alpha and beta) analyses. All the analyses have been executed on the feature-table (table that reports the number of feature-bacteria for each sequenced sample) evaluated by using the DADA2 pipeline[5] and the corresponding taxonomic composition of the samples has been based on the database "SILVA 132" (99% sequence similarity). In particular, alpha diversity index evaluates the richness of each sequenced sample, beta diversity 2 index indicates the dissimilarity between each pair of sequenced samples.[6] Due to the high complexity of the project and to the numerous pairwise comparisons, an ordination algorithm has been applied to the beta diversity dissimilarity matrix in order to reduce the dimensionality of the analysis to few principal components by means of the MultiDimensional scaling (MDS) approach called "Principal Coordinates Analysis" (PCoA). The corresponding results have been visualized with the "Emperor" tool visualizer.

One thousand samples have been sequenced for metagenomics and analysis performed on the first 500 samples. The results are as follows:

Alpha diversity. The analysis of the rarefaction curves on the Shannon index indicated a good sampling quality (Figure 2A) as the richness index does not increase significantly with the sampling depth for each subject. Moreover, a strong difference in the bacteria richness between pharynges and noses has been observed (Figure 2B), in line with previous reports.[7] Nostrils and pharynges host a flora consisting of bacterial belonging to 31 phyla. Among these, 5 phyla represented more than 99% of the total microbiota: Firmicutes, Proteobacteria, Actinobacteria, Fusobacteria and Bacteroidetes (Figure 2C).

Beta diversity. The beta diversity analysis was evaluated by using the Weighted Unifrac metric. Pharynges and nostrils cluster separately when plotted with the first three Principal Coordinates (Axis 1, Axis 2 and Axis 3) in PCoA [8]. Closer samples have smaller dissimilarity values than those ordered further apart. The statistical test on beta diversity Weighted Unifrac distances of each pharynx to all other pharynges and to all other noses was performed. The Permanova pairwise test presents a p-value smaller than 10-3 thus indicating significant differences in compositional structure between the two groups.

In addition, the beta diversity analysis also showed that the pharyngeal samples from patients undergone allogeneic HSCT were significantly different in compositional structure when compared to patients undergone autologous HSCT ($p<0.001$). A similar trend was also observed when nasal swabs were compared between the two groups of patients ($p=0.077$).

Metagenomics of the remaining samples is ongoing along with targeted metabolomics. In this regard, we have preliminary data showing the detectability of indole derivative[9] in both nasal 4 and pharyngeal samples (data not shown). This indicates that targeted metabolomics is highly feasible in the collected samples. Overall, the preliminary results indicate significant differences in microbial composition between nostril and pharynx as well as between patients at different risk for invasive aspergillosis.

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GENE THERAPY FOR HEMOGLOBINOPATHIES: FROM GENE REPLACEMENT TO GENOME EDITING

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Twenty-six years ago, genetically modified bone marrow cells were administered for the first time to a child suffering from adenosine deaminase deficiency, a rare disorder of the immune system. Since then, a series of authoritative clinical studies proved that transplantation of genetically modified hematopoietic stem cells can cure severe diseases like immunodeficiencies, lysosomal storage diseases and hemoglobinopathies, transforming gene therapy into a clinical reality. Gene therapy for beta-thalassemia and sickle cell disease is currently based on the use of lentiviral vectors to transfer a beta-globin gene under the transcriptional control of regulatory elements of the beta-globin locus. Preclinical and clinical studies have recently shown the safety and potential efficacy of this therapeutic approach. Nevertheless, a number of factors are still limiting its efficacy, such as quality and dose of hematopoietic stem cells, suboptimal gene transfer efficiency and gene expression levels, and toxicity of myeloablative regimens. In addition, the cost and complexity of the current vector and cell manufacturing technology severely limits its application in less favored countries, where hemoglobinopathies may reach endemic proportions. More recently, a new generation of gene editing technology emerged, aimed at correcting the genome rather than replacing defective gene function. This technology relies on designer nucleases capable of generating double-stranded breaks in genomic DNA, which are then repaired either by error-prone non-homologous end-joining or by the more precise homologous recombination. Genome editing may overcome at least some of the limitations of classical gene replacement and provide additional therapeutic opportunities, though its safety and efficacy are yet to be proven in the clinical reality.

MUTATIONS OF THE $P2Y_{12}$ RECEPTOR GENE ASSOCIATED WITH BLEEDING COMPLICATIONS

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Concomitant activation of both G-protein coupled receptors for ADP that are expressed on platelets, P2Y1 and $P2Y_{12}$, is necessary for normal platelet responses to ADP.[1] $P2Y_{12}$ contributes to the formation of platelet aggregates by two mechanisms: 1) PI3K–mediated inactivation of RASA3, the GTPase activating protein that switches $P2Y_1$/CalDAG-GEFI-activated Rap1-GTP back to Rap1-GDP,[2,3] and, 2) by inhibiting adenylyl cyclase, thereby counteracting the inhibitory effects on platelets of molecules that increase cyclic adenosine monophosphate (cAMP) levels, such as prostacyclin.[4] Therefore, $P2Y_{12}$ plays a central role in the formation of platelet aggregates both in physiological hemostasis and in pathological thrombosis: indeed, antagonists of $P2Y_{12}$ decrease the risk of major adverse cardiovascular events, but also increase the bleeding risk.[5]

The human $P2Y_{12}$ was identified and cloned in 2001: it contains 342 amino acid residues[6].v The P2RY12 gene maps to chromosome 3q21-q25.[7]

Inherited $P2Y_{12}$ deficiency, first described in 1992,[8] is characterized by lifelong history of excessive bleeding, prolonged bleeding time, rapidly reversible aggregation in response to ADP, even at very high concentrations.[8] Lack of inhibition by ADP of cAMP increase or phosphorylation of vasodilator-stimulated phosphoprotein (VASP) induced by stimulators of adenylyl cyclase (e.g., prostaglandin I_2 or E_1, forskolin, adenosine) is the most specific, confirmatory test of the disorder.[8] *P2YRY12* haplotypes associated with slight variations in platelet responses to ADP, but not with bleeding, have been described.

Six patients with severe deficiency of $P2Y_{12}$ have been described so far. Two of them displayed homozygous bp deletions in the encoding gene, which result in frameshifts (p.Gln98fs and p.Gly12fs) and premature truncation of the protein.[5,9] Two sisters had an identical single bp deletion (378delC) occurring just beyond the coding sequence for the third transmembrane domain in $P2Y_{12}$, resulting in a frame shift (p.Thr126fs) and premature truncation of the protein.[5,9] As only alleles encoding the mutated DNA sequence were found by PCR analysis, the patients were considered homozygous for the 378delC mutation. However, a subsequent study revealed that they suffer from $P2Y_{12}$ deficiency owing to partial or complete deletion of the *P2YR12* gene in one allele and to the 378delC mutation in the other allele. The molecular defect that is responsible for the severe deficiency of $P2Y_{12}$ in another patient is less well defined: one mutant allele contains a deletion of 2 bp within the coding region, resulting in a frameshift (p.Phe240fs) and early truncation of the protein, while the other allele does not display any mutation.[5,9] It is likely that this patient has an additional, as yet unknown, mutation in the other allele that silences the normal $P2Y_{12}$ gene, as suggested by the finding his platelets contained $P2Y_{12}$ transcripts derived from the mutant allele only and that his daughter, who had a heterozygous phenotype, inherited the mutant allele from her father and a normal allele from her mother. Finally, a patient from Japan was found to be homozygous for a single nucleotide substitution in the translation initiation codon (ATG to AGG), which resulted in complete failure to synthesize the $P2Y_{12}$ protein.[5,9]

Patients with dysfunctional $P2Y_{12}$ receptor have been described. One patient displayed a missense mutation in one allele, changing the codon for Arg256 to Gln, and a missense mutation in the other allele, changing the codon for Arg265 to Trp. Neither mutation interfered with the surface expression of the $P2Y_{12}$ receptor but both altered receptor function.[5,9] A heterozygous point mutation in the same region of the molecule (TM6/EL3), responsible for a p.Pro258Thr substitution, was described in a patient with a mild bleeding disorder and severely impaired ADP-induced platelet aggregation;[8,9] a patient with the same mutation was subsequently described by another group.[9] A heterozygous mutation, predicting a p.Lys174Glu substitution in $P2Y_{12}$, was identified in one patient.[8,9] Platelets from this patient showed reduced and reversible aggregation in response to ADP and an approximate 50% reduction in binding of [^{3}H]2MeS-ADP. One patient displayed a heterozygous mutation (p.Pro341Ala) in the PDZ binding sequence of $P2Y_{12}$, associated with

reduced expression and compromised recycling of $P2Y_{12}$.[9] In another patient, the expression of mutant p.Arg122Cys $P2Y_{12}$ was reduced, probably as a consequence of internalization, followed by subsequent receptor;[9] a heterozygous p.Arg122His mutation was described in a patient with mildly reduced $P2Y_{12}$ function.[9] Finally, two brothers were described with dysfunctional $P2Y_{12}$, normal expression of the receptor but decreased affinity for its ligand, associated with homozygous p.His187Gln substitution.[10]

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THE MULTIPLE FEATURES OF CLL TISSUE MICROENVIRONMENT

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Chronic lymphocytic leukemia (CLL) is the most common adult leukemia in the western world. The evolution and maintenance of CLL are strictly related to the ability of leukemic cells to invade and manipulate tissue microenvironments (TME). Despite an apparent long life in vivo, CLL cells undergo in spontaneous apoptosis in vitro, this is avoided by culturing CLL cells in presence of a feeder layer represented by stromal cells,[1] endothelial cells[2] or macrophage population (also called nurse-like cells-NLC).[3] The inefficient apoptosis is mainly due to overexpression of the antiapoptotic protein Bcl-2, due to early-onset genetic alterations and further increased by TME signals received by leukemic cells inside tissue niches.[4]

CLL cell interactions with the supportive TME play a critical role in the disease pathogenesis. CLL cells recirculate between peripheral blood and secondary lymphoid organs, were they proliferate in peculiar tissue areas, termed pseudofollicular proliferation centers. Homing to tissues is dependent on a tightly regulated interaction orchestrated by chemokines and chemokines receptors and adhesion molecules, involving signaling pathways involved in CLL homing, survival and proliferation.

The role of the surrounding normal TME cells (macrophages, T-cells, stromal cells, endothelial cells, dendritic cells) is multifaceted, determining an environment able of elicit CLL proliferation and viability (permissive microenvironment), sustaining and feeding leukemic cells (nurturing microenvironment) and, of importance, protecting CLL cells from immune surveillance and response (protective microenvironment), by determining a peculiar impairment of both adoptive and innate immunity. As consequence, CLL patients present a progressive immunodeficiency due to the ability of CLL cells to manipulate their TME escaping immunosurveillance and inducing immunosuppression.[5] CLL cells are accompanied by an expanded population of regulatory and exhausted T cells, and surrounded by NLC with M2 (immunosuppressive) properties. NLC share several features with tumor-associated macrophages (TAM), protect leukemic cells from undergoing spontaneous or drug-induced apoptosis in a contact dependent manner.[6]

In the last years, treatment of CLL has been revolutionized by the introduction of novel biologic-targeted drugs, including the tyrosin kinase inhibitors (TKI) ibrutinib and idelalisib and, more recently, the Bcl2 inhibitor Venetoclax, envisioning a possible shift towards a non-chemotherapy treatment era. Noteworthy, the molecular targets of ibrutinib and idelalisib are not restricted to CLL or B-cell compartment, but also regulate key functions of other cellular elements, i.e. NK, T cells, macrophages and osteoclasts modifying the nurturing and protective niches of B cell clone into tissues, altering the balance between a protective and permissive microenvironment.[7] Among cytoplasmic TKI of Tec family, macrophages express Btk, Tec and Bmx, that are targeted by ibrutinib. Btk function is involved in the phagocytosis and ibrutinib reduces the phagocytic ability of macrophages and increases the immunosuppressive profile of NLC exacerbating the expression of M2 markers, also protecting CLL cells from drug-induced apoptosis.[8] On the contrary, lenalidomide was shown to modify the balance of NLC phenotype from a M2-skewed immunosuppressive towards a M1-skewed inflammatory profile.[9] In T cells, three Tec kinases are expressed, Itk, Rlk/Txk, and Tec and ibrutinib may influence T cell function by skewing T cells from a Th2-dominant to a Th1 and CD8+ cytotoxic population, due to a selective Itk inhibition and a compensatory mechanism mediated by Rlk that remains uninhibited in Th1 cells. Moreover, in CLL patients ibrutinib enhances the persistence/expansion of activated T cells, reduces the number of Treg cells, increases Th17 CD4+ population also reversing the exhausted phenotype of T cell population.[10]

Of interest, in vitro studies indicate a possible TME role in Bcl-2 inhibition resistance mediated by BCR activation that protects CLL cells from venetoclax-mediated apoptosis by up-regulation of Mcl-1.[4]

Recently, most therapeutic strategies against cancer have focused on targeting TME, developing strategies to re-educate the TME and the immune response. In this sense, drugs that target the immune checkpoint blockade molecules, used as single agents or in combination are of potential interest in CLL treatment and are under investigation. At the same time, introducing high efficient engineered CAR-T cells in CLL treatment needs to keep in consideration the complexity of the immunologic impairment typical of CLL TME.

Although an important amount of knowledge have been acquired in the last few years, the impact of new drugs on CLL TME need to be better elucidated, in order to optimize the potential synergism of these drugs obtaining the better advantage of treatment combinations.

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IL-1R8, A NEGATIVE REGULATOR OF INFLAMMATION AND CHECKPOINT MOLECULE

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IL-1 family members are central mediators of inflammation and homeostatic differentiation and activation of immune cells. IL-1 receptor (ILR) and Toll Like Receptor (TLR) pathway activation is crucial for immune surveillance against infectious agents and sterile damages, but given its broad inflammatory potential it is tightly regulated through ligands with antagonistic or anti-inflammatory activity, or decoy and negative regulatory receptors.[1] IL-1R8 (also known as SIGIRR) is a members of the ILR family acting as a negative regulator of the IL-1 system. IL-1R8 dampens ILR- and TLR-mediated cell activation and is a component of the receptor complex recognizing the anti-inflammatory cytokine IL-37. IL-1R8-deficiency is associated with different pathologic conditions, ranging from infectious and sterile inflammation and autoimmunity, to cancer-related inflammation.[2] In the context of haematological neoplasia, IL-1R8-deficiency in the mouse is associated with increased lymphoproliferation and neoplastic transformation. In particular, in the mouse model of CLL (TCL1), IL-1R8 deficiency induced an earlier and more severe appearance of monoclonal B cell expansion and a reduced mouse life span, mimicking the aggressive variant of human CLL.[3] In agreement with these results in mice, human malignant B cells were shown to express lower levels of IL-1R8 mRNA than normal B cells. These results are in line with the role that both genetic defects and microenvironment stimuli, including endogenous TLR or ILR ligands, have in contributing to CLL development and progression. Natural killer (NK) cells are innate lymphoid cells which mediate resistance against pathogens and contribute to the activation and orientation of adaptive immune responses. NK cells mediate resistance against haematopoietic neoplasms but are generally considered to play a minor role in solid tumour carcinogenesis. IL-1R8 was recently shown to serve as a checkpoint for NK cell maturation and effector function. Its genetic blockade unleashes NK-cell-mediated resistance to hepatic carcinogenesis, haematogenous liver and lung metastasis, and cytomegalovirus infection. These results indicate that IL-1R8 acts as a novel checkpoint molecule of NK cells, tuning NK cell anti-tumor and antiviral activity.[4]

Thus, IL-1R8 contributes to the delicate equilibrium between host defense and detrimental inflammation, cancer-related inflammation and immunesurveillance of cancer.

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BEST ABSTRACTS

BO001

GENOTYPING OF PLASMA CELL DISEASE ON THE LIQUID BIOPSY

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Introduction. Easily accessible, real-time genotyping is desirable for patients suffering from plasma cell (PC) disorders for diagnostic, prognostic and therapeutic purpose. Circulating cell-free DNA (cfDNA) might be an accessible source of tumor material in patients with PC diseases to identify cancer-gene somatic mutations. Accessing the peripheral blood (PB) has clear advantages in terms of the sampling procedure itself, and has the potential to better reflect tumor heterogeneity.

Methods. The study was based on a series of 30 consecutive patients with PC disorders, of whom the following material was collected: (1) cfDNA isolated from plasma, (2) tumor genomic DNA (gDNA) from CD138+ purified bone marrow (BM) PCs for comparative purposes, and (3) normal germ line gDNA extracted from PB granulocytes, to exclude polymorphisms. A targeted resequencing gene panel including coding exons and splice sites of 14 genes was specifically designed to allow a priori the recovery of at least one clonal mutation in 68% of MM patients. Ultra-deep next-generation sequencing (NGS) of the gene panel was performed on MiSeq (Illumina) using the CAPP-seq library preparation strategy (NimbleGen). The somatic function of VarScan2 was used to call non-synonymous somatic mutations, and a stringent bioinformatic pipeline was developed to filter out sequencing errors. The sensitivity and specificity of plasma cfDNA genotyping were calculated in comparison with tumor gDNA genotyping as the gold standard.

Results. Circulating cfDNA was detectable in plasma samples with a median of 10523 haploid genome-equivalents/mL. The application of our targeted ultra-deep NGS approach for plasma cfDNA genotyping resulted in ≥90% of the target region covered >1000X in all plasma samples, and >2000X in 25/30. Overall, 20/30 (67%) patients harbored somatic mutations that were detectable in plasma cfDNA. Quite consistent with the typical spectrum of mutated genes in MM, plasma cfDNA genotyping revealed somatic variants of NRAS in 27%; KRAS in 20%; TP53, TRAF3 and FAM46C in 10%, respectively; CYLD, DIS3 and BRAF in 7%, respectively; and IRF4 in 3% of cases. cfDNA genotyping correctly identified 71% of mutations (n=32/45) discovered in tumor PCs and overall the variant allele frequencies in plasma samples correlated with those in tumor biopsies. Notably, the remaining mutations not discovered in cfDNA had a low representation in the purified BM PCs. ROC analysis showed that cfDNA genotyping had the highest detection rate (100%) if mutations were represented in >5.78% of the alleles of the purified BM PCs. In none of the cases, cfDNA genotyping identified additional somatic mutations not detected in the purified BM PCs.

Conclusions. Our results provide the proof of principle that circulating tumor cfDNA genotyping is a feasible, non-invasive real-time approach that reliably detects clonal and subclonal somatic mutations represented in at least 6% of alleles in tumor PCs.

BO002

THE GENOMIC LANDSCAPE OF STRUCTURAL VARIATIONS AND COMPLEX EVENTS IN MULTIPLE MYELOMA

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Introduction: Multiple Myeloma (MM) initiation and progression is driven by recurrent cytogenetic events, *i.e.* multiple trisomies or translocations within the immunoglobulin locus. Gene mutations have been extensively studied, and they are generally involved in late phases of disease development. On the contrary, very little is known about structural variations (SV), which are increasingly emerging as critical driver in several cancers.

Methods: We performed whole genome sequencing (WGS) on 67 CD138+ purified bone marrow MM samples from 30 patients (median of 2 samples per patient; range 1-4), to which we added 22 previously published cases (Chapman et al, Nature 2011) for a total of 89 tumour samples. We defined SVs as inversions, translocations, internal tandem duplications and deletions, which we analysed using publicly available tools developed at the Wellcome Trust Sanger Institute. Events with > 3 independent SVs involved in distinct copy-number abnormalities (CNAs) were defined as "complex".

Results: We found a stunning 1887 unique SVs in the whole cohort, with a heterogeneous distribution across the entire series (median 29 per patients, range 0-156). IGH and MYC translocations were the most frequent recurrent events and accounted for only 5.3% of the entire SV catalogue. Integrating data on SV and CNAs we found that a SV was responsible for ~90% of CNAs where a breakpoint could be mapped. Furthermore, 93% of patients carried multiple CNAs across different chromosomes all sustained by the same complex SV. Our analysis thus offers a pathogenic explanation of many recurrent CNAs in MM.Overall, 136 complex events were observed in 43/52 patients (83%). We found 34 instances of chromotripsis (Korbel J.O. *et al*., Cell 2013) in 18/52 patients. The vast majority (30/34) were clonal and conserved during evolution, suggesting a potential early role in MM pathogenesis. In addition, we observed 5 chromoplexy (Korbel J.O. *et al*., Cell 2013) events acquired in 5 patients. More interestingly, in 13 patients we found an entirely novel complex event characterized by multiple concatenated translocations causing small CNAs on more than 2 different chromosomes, that we named template insertion (TI). Interestingly, 77% of TIs resulted in a translocation involving an important MM oncogene (8 MYC and 2 CCND1), suggesting that this is a novel relevant driver mechanism in MM. Reconstructing their order of acquisition, we show that SVs and complex events are at the nodes of initiation and subclonal diversification during MM life history, with high variability of timing from patient to patient, confirming their driver role in MM pathogenesis.

Conclusions: In this study, we described for the first time the landscape of MM SV and complex events, showing their critical role in MM pathogenesis. Overall these data suggested a new pathogenetic model where MM evolution is driven by few clonal sweeps promoted by heterogeneous and private structural events.

BO003

THE SETBP1 ONCOGENE INDUCES THE TRANSCRIPTION OF A NETWORK OF DEVELOPMENT GENES BY ACTING AS AN EPIGENETIC HUB

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Introduction: SETBP1 variants occur as somatic mutations in several hematological malignancies such as atypical Chronic Myeloid Leukemia and as *de novo* germline mutations in the Schinzel-Giedion Syndrome (SGS), a disorder characterized by severe intellectual disability and multi-organ development abnormalities.

Methods: ChIP-Seq, RNA-Seq, Co-Immunoprecipitation, HPLC-Mass Spectrometry, ATAC-Seq, in-utero electroporation.

Results: ChIP-Seq experiments performed on 293 FLP-In lines harboring WT and mutated (G870S) SETBP1 led to the identification of 3065 genomic regions bound by SETBP1-G870S. These regions were enriched in A/T nucleotides ($p<0.0001$), which suggested that SETBP1 could bind gDNA through its AT-hook domains. RNA-Seq analyses of

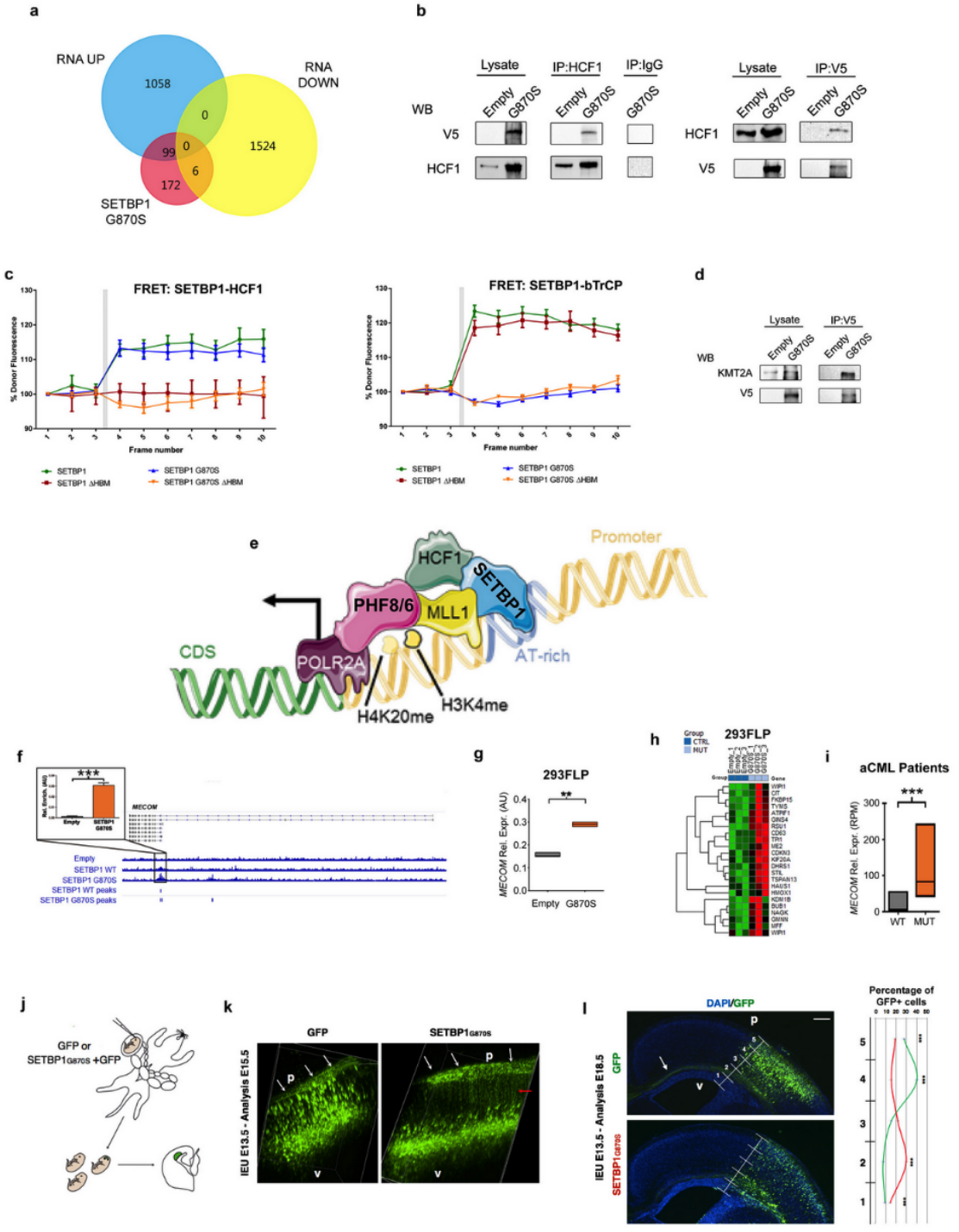

Figure 1.

G870S vs Empty control identified 2687 differentially expressed genes (DEGs); of them 57% were down- and 43% up-regulated. The intersection between genes bound by SETBP1 in promoter regions and DEGs (FDR<0.001) revealed 105 co-occurring genes. The vast majority of these genes was upregulated (99; 94.3%; $p<1x10^{-6}$), suggesting a role for SETBP1 as a positive inducer of gene expression (Figure 1a). Deletion of AT-hook regions 1/2 (Del-AT1/2) led to a complete normalization of the expression level of SETBP1 target genes and loss of SETBP1-gDNA interaction, confirming that this interaction relies on functional AT-hooks. Co-immunoprecipitation/proteomics and FRET experiments (Figure 1b,c,d) revealed a direct interaction between SETBP1, KMT2A (MLL1), PHF8/6 and HCF1 (Figure 1e). Linear domain analysis suggested the presence of a HCF1 binding motif (HBM) at position 991-994 of SETBP1. Deletion of this region caused the abrogation of SETBP1/HCF1 interaction and normalization of the expression level of SETBP1 target genes.

ChIP-Seq and ChIP data (Figure 1f) showed that SETBP1 binds to the MECOM promoter. In line with these findings, MECOM and MECOM target genes were differentially expressed (p<0.0001) in cells expressing SETBP1-G870S (Figure 1g,h). Similar results were obtained in 32 aCML cases (11 positive and 21 negative for SETBP1 somatic mutations), where SETBP1-positive patients expressed higher levels of MECOM (p=0.0002. Figure 1i) and MECOM target genes (p<0.0001).

Transduction of radial glial progenitors of cerebral cortices of E13.5 mouse embryos with an expression plasmid encoding SETBP1-G870S using an in utero electroporation system (Figure 1j) caused an impairment of mouse embryo neurogenesis and a profound delay in neuronal migration (Figure 1k,l), which was restored by transduction with a Del-AT1/2 vector, indicating that SETBP1 epigenetic machinery is directly responsible for the neuroanatomical defects described in SGS.

Conclusions: In summary, this work unveils for the first time a new SETBP1 function which directly promotes gene transcription and clarifies a new mechanism operating in myeloid malignancies and in SGS.

BO004

LONG-TERM OUTCOME OF A COHORT OF ELDERLY ACUTE MYELOID LEUKEMIA PATIENTS, NOT ELIGIBLE FOR ALLOGENEIC STEM CELL TRANSPLANTATION, INFUSED WITH ALLOREACTIVE NATURAL-KILLER CELLS AS CONSOLIDATION THERAPY

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Background: Several studies revealed the potential of adoptive immunotherapy with alloreactive natural Killer (NK) cells in acute myeloid leukemia (AML) patients. Adoptively transferred NK cells can be successfully expanded *in vivo* and significantly impact on leukemic cells killing.

Aims: Aim of this work is to establish the impact on long-term survival and relapse rate of adoptive immunotherapy with alloreactive NK cells as post consolidation therapy in a cohort of AML patients in complete remission (CR).

Methods: Seventeen AML patients, with a median age of 64 years (range 53-73), were enrolled. Patients were in first CR after standard chemotherapy regimens and were unfit for allogeneic stem cell transplantation (ASCT). Patients in morphologic or better CR with a haploidentical KIR-L–mismatched donor, received NK cells after an immunosuppressive chemotherapy regimen based on fludarabine 25 mg/mq (day-7 to-3) and cyclophosphamide 4 g/mq (day-2). Two days after cyclophosphamide administration, patients received NK cell infusion (day 0), which was followed by subcutaneous administration of IL-2 ($10x10^6$ IU/day, 3 times weekly) for 2 weeks (6 doses total). To correlate donor NK cell activity with clinical response, donor NK cells were assessed before and after infusion.

Results: NK cell infusion was well-tolerated and no signs of GVHD were described. The median follow-up is now extended to 55.5 months (range 6-125 months) vs 22.5 (range 6-68 months) in the original publication. 8 out of 16 evaluable patients (50%) are alive disease-free. Among relapsing patients (8/16), median time to relapse was 9 months (range 5-51 months). Three of the relapsed patients maintained a prolonged CR for 15, 24 and 51 months, respectively. The patient who relapsed after 51 months received a second NK infusion, thus obtaining a second CR. All patients treated with molecular disease achieved molecular CR. Based on these data, 11 out of 16 (69%) patients were considered as responders, whereas 5 out of 16 (31%) were non-responders. These long-term clinical results were compared with the outcome of patients from a historical control cohort, treated with standard chemotherapy regimen and who did not receive NK immunotherapy. In the control group, 14 out of 15 patients (93%) relapsed, with a median time to relapse of 11 months (range 3-79). Due to the low numbers of evaluable patients, the difference in terms of DFS is not statistically significant, although a trend toward an increase of DFS for patients who received NK infusion may be observed. In agreement with our previous work, the predictive impact of higher alloreactive NK donor repertoire on clinical outcome was confirmed.

Conclusion: Adoptively transferred alloreactive NK cells have the potential to induce prolonged control of AML in the non transplant setting. Moreover, the composition of NK graft in terms of frequency of alloreactive NK cells is likely to influence the long-term clinical response.

BO005

EXPLOITING CUTTING-EDGE TECHNOLOGIES TO ANALYZE LOSS OF HLA IN A MULTICENTRIC COHORT OF POST-TRANSPLANTATION RELAPSES: RESULTS FROM THE HLALOSS GLOBAL COLLABORATIVE STUDY

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Introduction. Genomic loss of the patient-specific HLA has been described in previous single-center studies as a frequent mechanism by which leukemic cells evade immune control and outgrow into a clinically evident relapse. HLA loss is deemed to account for up to 30% of relapses after HLA-haploidentical transplants, but the actual frequency and clinical relevance of this phenomenon in other transplantation settings are largely unknown. Here we present the first

global collaborative study to investigate the incidence of this phenomenon across transplant platforms.

Methods. Twenty transplant centers from across the globe (Europe n=16, North America n=3, Asia n=1) joined to form the HLALOSS consortium. To date, we collected a total of 565 cases of relapse from adult patients with acute leukemias, myelodysplastic syndromes or myeloproliferative neoplasms after allogeneic HSCT from HLA-haploidentical relatives (31.7%), HLA-mismatched unrelated donors (MMUD, 20.9%), 10/10-matched unrelated donors (MUD, 38.0%), or unrelated cord blood units (UCB, 9.3%). Where available, the donor and patient germlines and the patient pre-transplant disease were tested in parallel. Cases were analyzed using conventional HLA typing of sorted leukemic blasts, the recently developed HLA-KMR assay (Ahci and Toffalori, Blood, 2017) or a novel Next-Generation Sequencing method developed to cover all possible HLA-A,B,C,DRB1,DQB1 and DPB1 alleles and to analyze multiple samples in a single run.

Results. To date, we analyzed 368 cases of post-transplantation relapse after haploidentical (n=151), MMUD (n=102), 10/10-matched, HLA-DPB1 mismatched MUD (n=86), or UCB (n=29) HSCTs. Of these, 274 cases were analyzed using the newly developed HLA sequencing platform. This method resulted particularly robust, reliable and sensitive in analyzing large sample series: with a minimum read-depth of 1000x, up to 0.5% of target DNA could be detected in artificial chimerism curves, and relapse samples tested in parallel via the sequencing platform and HLA-KMR (n=10) showed remarkable concordance between the two methods ($R2=0.86$, $p<0.0001$). In the total 368 cases analyzed to date by the different methods, we have detected 55 HLA loss post-transplantation relapses, 39 of which after haploidentical HSCT (25.8% of relapses in this setting), 12 after MMUD HSCT (11.8%), 4 after 10/10 MUD HSCT (4.7%) and, noticeably, none after UCB HSCT.

Conclusions. The present data, obtained from the largest collaborative study on the immunobiology of relapse to date, confirm the clinical relevance of HLA loss as a major mechanism of immune evasion and post-transplantation relapse, including after HSCT from partially HLA-incompatible unrelated donors. The only exception is represented by UCB HSCT which, despite being often performed across multiple major HLA incompatibilities, do not appear to be associated to this relapse modality.

ORAL COMMUNICATIONS

Acute Leukemia 1

CO001

THE DIAGNOSTIC AND STRATIFICATION APPROACH FOR PATIENTS RECRUITED IN THE AIEOP-BFM ALL 2017 PROTOCOL FOR PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA IN ITALIAN (AIEOP) CENTERS: PRELIMINARY RESULTS

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Introduction. Next Generation Sequencing (NGS) methods, contributed to the understanding of the important relationship between prognostic markers and personalized therapy, in a cost- and time-effective manner. In the new protocol AIEOP-BFM ALL2017, starting in 2018, an early stratification of patients is needed to investigate the efficacy of therapies directed on specific genetic lesions. The main goal of diagnostics and monitoring in new protocol AIEOP-BFM-ALL 2017 is to provide a rapid and multi-comprehensive strategy for clinical decision making, in particular for actionable lesions and precision medicine.

Methods. NGS was developed within the Euroclonality-NGS consortium to identify clonal IG/TR gene rearrangements for MRD quantification at day +33, with purpose-build bioinformatics tools ARResT/Interrogate and ViDjil for IG/TR NGS-datasets. NGS-digital-MLPA (dMLPA) has been developed to recognize the 'IKZF-plus' patient subgroup by day +33. Moreover, multiplex-RT-PCR used in routine diagnostics for known fusion genes, has been improved to detect TCF3-HLF/t(17;19) transcripts associated to poor prognosis and needing early intensive therapy. In addition, the probe-based RNA Target-Capture NGS has been developed to identify translocations of recurrent genes with any partner gene, by using the Trusight RNA Pan Cancer Library Prep targeting 1385 cancer-associated genes (Illumina). Bioinformatics strategy has been applied for fusion gene detection NGS-datasets, employing both ready-to-use web-based platform (BaseSpace, Illumina Cloud), as well as in-house bioinformatics method, named BreakingPoint.

Results. Since May 2017, we analyzed 226 patients; IG/TR NGS screening identified more markers than conventional methods. Indeed, 1439 IG/TR rearrangements were identified by NGS, with a mean of 6.37 clones/pt (range 0-15), with a mean response time of 14.6 days from diagnosis (range 7-26 days). In 5 out of 226 (2.2%) cases, no IG/TR markers were identified: 3/5 were very immature T-ALL and 2/5 were BCP-ALL (1 BII in addition tp 1 cases with 2.5% blasts). A total of 86 samples was analyzed in parallel with conventional MLPA and dMLPA to detect Ikaros-plus patients, obtaining 98.8% concordance (only 1/86 discordant) and overall 85% concordant results on Copy Number Variation analysis. In a cohort of 261 patients, selected by either MRD at TP1 (d33) ≥5x10-4 or relapse, RNA-targeted analysis detected 109 fusions, involving recurrent genes such as ETV6, NUP214, BCL9, EBF1, MLL, TCF3 (two cases with TCF3/HLF), ZNF384, PAX5 and JAK2.

Conclusions. NGS allows to identify translocations as well as IG/TR rearrangements, while digital MLPA detects copy number alterations (CNAs) associated to Ikaros-plus. By this combined new methods, in addition to routine diagnostics, it's possible to fine-tune risk stratifications and treatment for genetically defined subgroups, for which a specific experimental arm will be available within a controlled clinical protocol.

CO002

NOVEL MUTATIONS AND TRANSLOCATIONS INVOLVING NUCLEOPHOSMIN (NPM1) GENE IN ACUTE MYELOID LEUKEMIA (AML) AND LEADING TO ABERRANT CYTOPLASMIC NPM1

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Background: Nucleophosmin (NPM1) gene mutations occur in 50–60% of adult AML with normal karyotype. About 50 NPM1 mutations have been so far identified, all clustering in exon-12 but few sporadic cases involving either exon-9 (one) (Mariano *et al.*, Oncogene 2006) or exon-11 (two) (Albiero *et al.*, Leukemia 2007). More recently, rare NPM1 fusion proteins have been also described in AML (Campreger *et al.*. Haematologica, 2016). In spite of molecular heterogeneity, all mutations cause common changes at the C-terminus of NPM1 mutants, *i.e.* loss of tryptophans 288 and 290 (or 290 alone) and creation of a new nuclear export signal (NES) motif, which binds to Exportin1/Crm1 and mediates its aberrant accumulation in the cytoplasm of leukemic cells, an event which is detectable by immunohistochemistry.

Aims: Here, we aimed to identify novel NPM1 genetic lesions, either mutations or NPM1 fusion proteins, leading to aberrant cytoplasmic NPM1 in AML, a critical event in leukemia development and maintenance (Brunetti *et al.*, ASH Abs 877, 2017).

Methods: We applied a combinatorial approach of immunohistochemistry (IHC), western blot (WB) with specific antibodies for either the mutated or the wild-type NPM1 protein, NPM1 gene sequencing and RNAseq to a large number (about 900) of patients from either our center or other Italian centers and the MLL laboratory in Munich, and focused on the discordant cases for IHC, WB or gene sequencing to identify novel NPM1 genetic lesions. In the newly discovered NPM1 mutated genes/fusion transcripts, search for acquisition of a NES motif in their protein sequence and strength of the NES were functionally evaluated in an overexpression ectopic cellular system.

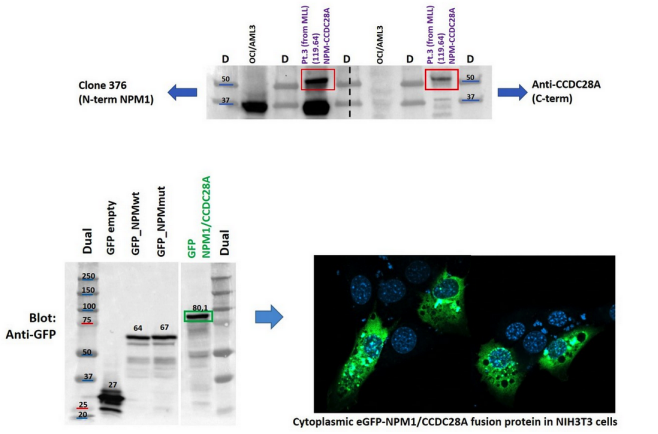

Figure 1.

Results: This research approach confirmed the rarity of mutations involving exons other that exon 12 in NPM1-mutated AML, and allowed the discovery of different novel mutations in exon 6 (n=3, Italian center) and exon 5 (n=1, MLL laboratory), which we have previously reported (Rossi R *et al.*, SIES 2016). Here, RNAseq analysis (MLL laboratory) lead to identification of 3 novel NPM1 gene translocation involving i) RPP30, t(5;10)(q35;q23); ii) SETBP1, t(5;18)(q34;q12); and iii)

CCDC28A, t(5;6)(q35;q23), as partners of NPM1. Strikingly, in all cases the predicted new protein sequence while losing the C-terminus of wt NPM1 (including Trp 288 and 290) harbored an additional NES domain, either newly created or present in the partner protein sequence, which ensured its cytoplasmic accumulation, confirming the concept that NPM1 mutation in AML are 'born to be exported' (Bolli *et al.*, Cancer Res 2007). The NPM1/CCDC28A fusion protein, found also in one of our patients, is shown in Figure 1.

Conclusions: Our observations further support the view that cytoplasmic NPM1 dislocation is a critical event in leukemogenesis (Brunetti *et al.*, ASH Abs 877, 2017) and that immunohistochemistry, that detects, through cytoplasmic dislocation of NPM, 'all types' of NPM1 mutations, might be used as first step for directing further molecular studies.

CO003

FLT3 INHIBITORS DRIVE CLONAL TRILINEAGE TERMINAL DIFFERENTIATION OF NPM1-MUTATED/FLT3-ITD AML MIMICKING HEMATOLOGICAL RESPONSE

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Background: Acute myeloid leukemia (AML) is a heterogeneous disease characterized by a landscape of mutated genes including preleukemic and leukemic gatekeeper mutations. In the past years, new drugs, including a wide range of small molecule inhibitors, have been developed. Among them, the FLT3 inhibitor midostaurin and the IDH2 inhibitor enasidenib have been recently FDA-approved and, nowadays, are widely used in either 'clinical trials' or 'real-world' AML patients. Changing of the therapeutic landscape in AML is challenging the response criteria definition.

Hypothesis and Aims: The effects of specific small molecule inhibitors on leukemic cells, precisely because of their specific mechanism of action, can differ greatly from the cytotoxic effects of standard chemotherapy. Indeed, similarly to what observed in acute promyelocytic leukemia (APL) with all-trans-retinoic acid (ATRA) and arsenic trioxide (ATO), terminal differentiation of leukemic cells towards neutrophils has been reported with the use of different FLT3 inhibitors. Notably, most of these patients were given as responders. How this phenomenon, characterized by the persistence of FLT3-ITD in mature polymorphonuclear cells could be reconciled with the complete responses, defined by the European Leukemia Net(ELN) criteria, observed in the same patients has not been investigated.

Results: Here, we report three cases of NPM1mut/FLT3ITD AML where treatment with different FLT3 inhibitors, either alone (two: gilteritinib and sorafenib) or in combination with chemotherapy (one: midostaurin), induced clonal myeloid - including granulocytic, but also megakaryocytic and erythroid - terminal cell differentiation with peripheral hematological recovery which mimicked complete response according to the ELN-defined criteria. These conditions could be easily recognized through integration of the classical morphological and immunophenotypic analyses with other standard assays such as immunohistochemistry, revealing cytoplasmic NPM1 and mutated NPM1 at single cell level, with specific antibodies, and DNA fragments analysis by capillary electrophoresis, allowing the variant allelic frequency evaluation of NPM1 and FLT3 mutated genes (Figure 1).

Conclusions: Clonal trilineage myeloid terminal differentiation of leukemic cells in patients with AML undergoing treatment with FLT3, and possibly other, specific inhibitors is an under-recognized phenomenon of which clinician hematologists should be warned to properly evaluate response to therapy. Moreover, the fact that these 'terminally differentiated' cells are not recognized as 'leukemic' by immunophenotypic analysis raises also doubts over the appropriateness of flow cytometry as approach for the evaluation of MRD in this setting, an issue that will require to be addressed in clinical trials. Thus, our observation increase the challenges related to the most proper methodological approaches, besides the standard, to assess the response and response criteria definition in AML.

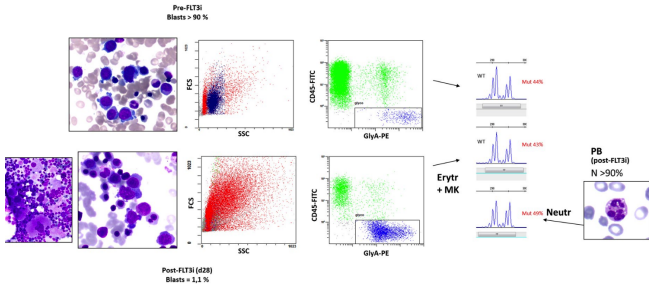

Figure 1.

CO004

INCIDENCE AND CLINICAL IMPACT OF OCCULT CENTRAL NERVOUS SYSTEM DISEASE IN ADULT WITH ACUTE LYMPHOBLASTIC LEUKEMIA. A MULTICENTER, ITALIAN STUDY. A REPORT FROM THE CAMPUS ALL NETWORK

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Introduction. In a modern context of improved management of acute lymphoblastic leukemia (ALL), central nervous system (CNS) involvement at diagnosis remains an obstacle to a long-term cure. We have previously reported that flow cytometry (FCM) is better than conventional cytology (CC) in demonstrating the presence of leukemic cells in the patients' (pts) cerebrospinal fluid (CSF), especially in samples with low cell counts. In the framework of the national Campus ALL program aimed at improving the management of adult ALL patients in the context of the GIMEMA protocols, in the present study we retrospectively evaluated the incidence of occult CNS positivity and its impact on outcome in 214 adult pts with newly diagnosed ALL from 11 centers.

Methods. Eighty-nine patients (42%) were females and 125 (58%) males, with a median age of 44 years (range 17-80), a median white blood cell (WBC) count of 10.6×10^9/L (range 0.1-457). One hundred and sixty-six pts (77%) had B-lineage ALL. The cytogenetic/genetic information was available in 157 (73%) pts: 51 (32%) had a BCR/ABL rearrangement, 30 (19%) a complex karyotype and 7 (4%) a MLL rearrangement. Pts were treated according to the GIMEMA/NILG ALL protocols or with the Hyper-CVAD program. Ninety-nine pts received an allogeneic stem cell transplant (ASCT). All CSF samples were double checked by CC and FCM. The presence of ≥10 clonally restricted or

phenotypically abnormal events was regarded as a FCM positivity. Based on the results of CSF examination, three different categories were recognized: manifest CNS+ (CC+FCM+), occult CNS+ (CC-FCM+) and CNS- (CC-FCM-), respectively.

Results. Overall, 18 (8%) pts had manifest CNS+, 39 (18%) occult CNS+ and 157 (74%) were CNS-. Median age, WBC count, B/T lineage, cytogenetic/genetic features did not differ significantly between the three categories. Complete remission (CR) was achieved in 171 (83%) pts, 9 (4%) died early in induction and 101/171 (59%) experienced a relapse. The frequency of CR rate did not vary significantly across the three identified categories. In univariate analysis, the CNS status correlated significantly with the incidence of relapse (p=.002) and with censor (=.0002). The four-year-overall survival (OS) for manifest CNS+, occult CNS+ and CNS- pts was 7%, 10% and 45%, respectively (p=.009) (Figure 1); no difference in terms of OS was observed between manifest and occult CNS+ pts (p=.17). In multivariable analysis, it was confirmed that a baseline status of manifest or occult CNS+ was independently associated with a higher relapse rate (p=.005).

Conclusions. Our large, multicenter study showed that in adult pts with ALL, FCM allows to detect occult CNS disease, even in conditions of low spinal fluid leukemic count; 2) the presence of occult CNS disease anticipates an adverse outcome. Further prospective studies on larger series are needed to confirm these data.

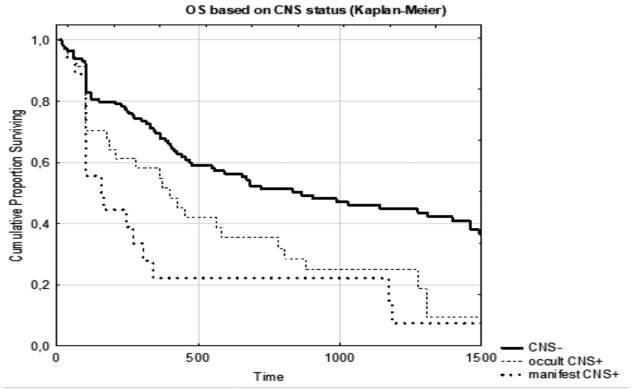

Figure 1.

CO005

BCR/ABL1-LIKE PROFILE HAS A NEGATIVE PROGNOSTIC IMPACT ON THE SURVIVAL OF ADULT B-LINEAGE ACUTE LYMPHOBLASTIC LEUKEMIA: FIRST ANALYSIS FROM THE GIMEMA LAL1913 PROTOCOL

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Introduction. In B-lineage acute lymphoblastic leukemia (B-ALL) without major fusion genes (*i.ei.e.* BCR/ABL1, ETV6/RUNX1, TCF3/PBX1 and KTM2Ar, defined as B-NEG ALL), the outcome is negatively influenced by the BCR/ABL1-like profile. We recently described a new method to identify BCR/ABL1-like cases by using a Q-RT-PCR approach coupled with an algorithm named "BCR/ABL1-like predictor" (Chiaretti *et al..,* 2018). In this study, we aimed at evaluating: i) the prognostic role of the BCR/ABL1-like profile within B-NEG ALL cases enrolled in the pediatric-oriented, minimal residual disease (MRD)-driven trial GIMEMA LAL1913 front-line protocol for adult BCR/ABL1-negative ALL, and ii) the features of BCR/ABL1-like cases.

Methods. Ninety-six B-NEG ALL (median age: 38.2 years, range 18.2-64.7) patients enrolled in the GIMEMA LAL1913 were screened for the BCR/ABL1-like profile, CRLF2 expression levels (Chiaretti *et al..*, 2016), JAK/STAT and RAS pathway mutational status (Truseq custom amplicon, Illumina MiSeq). The BCR/ABL1-like status was correlated with clinico-biologic variables (age, gender, WBC count, genetic features), complete remission (CR) achievement, disease-free and event-free survival (DFS, EFS) at 12 months.

Results. We identified 28 (29.2%) BCR/ABL1-like cases. CRLF2 overexpression was found in 19 cases (19.6%) and was associated with a BCR/ABL1-like signature (35.7% BCR/ABL1-like vs 13.2% of non-BCR/ABL1-like ALL, p=0.015). Similarly, JAK/STAT pathway mutations were more frequent in BCR/ABL1-like than in non-BCR/ABL1-like cases (33.3% vs 11.2%, p=0.032); RAS mutations did not significantly differ between the two subgroups. The BCR/ABL1-like status was strongly associated with outcome. Indeed, there was a trend towards an inferior CR rate in BCR/ABL1-like compared to non-BCR/ABL1-like cases (75% vs 90.8%, p=0.078). Consistently, MRD evaluation - feasible in 66 patients at TP2 - revealed that 52.9% of BCR/ABL1-like cases and 18.4% of non-BCR/ABL1-like were MRD-positive (p=0.009). Survival analyses showed that BCR/ABL1-like patients had a significantly inferior EFS and DFS than non-BCR/ABL1-like patients (45.2% vs 75.2%, p=0.005 and 60.3% vs 82.9%, p=0.039, respectively). In a multivariate model for EFS, the only variables that retained statistical significance were the BCR/ABL1-like profile (p=0.008, HR=2,58 CI 95% 1.28-5.21) and age (p=0.002, HR=1.03, CI 95% 1.01-1.06).

Conclusions. This first analysis of the GIMEMA LAL1913 protocol shows that BCR/ABL1-like ALL accounts for 29.2% of cases and highlights that this ALL subset is characterized by a lower CR rate, EFS and DFS, as well as MRD persistence also in a pediatric-oriented and MRD-driven clinical trial. These results retained statistical significance also in a multivariate analysis, thus corroborating the notion that the BCR/ABL1-like status should be evaluated at diagnosis to allow a more refined risk-stratification and to envision alternative treatment strategies upfront.

CO006

A NEW HUMANIZED MONOCLONAL ANTIBODY AGAINST A SPECIFIC GLYCOSYLATED EPITOPE OF CD43 FOR THE THERAPEUTIC TARGETING OF ACUTE LYMPHOBLASTIC LEUKEMIA T (T-ALL)

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Introduction: Acute lymphoblastic Leukemia T (T-ALL) accounts for about 20% of ALL cases. Currently, about 25% of pediatric and 50% of adult patients fail to benefit from standard chemotherapy; novel treatments are, therefore, eagerly awaited. Targeting of tumor-associated antigens by monoclonal antibodies (mAb) is one of the most effective immunotherapeutic strategies. We first developed a murine mAb, directed against a specific glycosylated epitope of CD43 (CD43/UN1) which demonstrated high reactivity against cortical thymocytes and T-ALL primary blasts and cell lines. Here we report the activity of the new humanized mAb (UMG1), and its afucosylated-engineered version (a-UMG1), in preclinical models of T-ALL.

Methods: CD43/UN1 expression was assessed by flow-cytometry on T-ALL and other tumor cell lines as well as on blood samples derived from healthy donors and T-ALL patients. A tissue microarray of human normal tissues was screened by immunohistochemistry according to

FDA/CE guidelines. Antibody-dependent cell-mediated cytotoxicity (ADCC) and cellular phagocytosis (ADCP) on primary T-ALL cells and T-ALL cell lines were evaluated by flow cytometry. Orthotopic and subcutaneous *in vivo* models on NSG mice (with or without administration of NK-92-CD16+ effectors) were used to evaluate the activity of both mAbs.

Results: By screening cancer cell lines and normal tissues, we found that CD43/UN1 expression was restricted to malignant T-ALL cells. We then evaluated 43 T-ALL patient-derived blasts and we observed specific CD43/UN1 binding in the cortical T-ALL (EGIL T3) patient subgroup. Therefore, to investigate ADCC or ADCP, T-ALL cells were cultured in the presence of peripheral blood mononuclear cells (PBMCs) or macrophages, at increasing mAb concentrations. Both UMG1 and a-UMG1 mAbs induced on NK cells CD16 downregulation, IFN-g production and degranulation (evaluated as percent increase of CD107+ cells) as well as cytotoxicity on T-ALL cell lines/primary blasts. All these effects were more evident with a-UMG1. Additionally, both mAbs induced ADCP. We demonstrated activity of both mAbs in different *in vivo* models of T-ALL. In the orthotopic model we observed 25% of treated mice free of disease after 100 days from injection as compared to 0% of control group. In subcutaneous model, we observed that our antibodies were effective in delaying tumor growth and in increasing mice survival. Of note, the injection of NK-92-CD16+ cell line strongly improved the anti-T-ALL activity of a-UMG1 mAb indicating ADCC occurrence *in vivo*. Moreover, we investigated CD43/UN1 modulation by several cytotoxic agents. Interestingly, methotrexate, doxorubicin (but not dexamethasone or vincristine), alone or in combination, increased antigen expression, and this effect led to improved ADCC.

Conclusions: Our findings indicate that UMG1 and a-UMG1 represent novel promising immune-therapeutic tools for the treatment of T-ALL patients.

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CO007

RNA-TARGET CAPTURE NEXT GENERATION SEQUENCING TO IDENTIFY NEW FUSION GENES IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

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Introduction. It is widely acknowledged that ALL is caused by acquisition of mutations, with a central role for chromosomal translocations. Therefore, genomic profiling of ALL has the potential to identify important new prognostic markers and druggable fusion genes. New advanced technologies methods contribute to understand the important relationship between prognostic markers and personalized therapy, in a cost- and time-effective manner. In this context, we propose Next-Generation Sequencing (NGS) as an efficient diagnostic tool to discover new and known prognostic genetic alterations in ALL patients using a single technological platform.

Methods. RNA-target-NGS has been setup to identify translocations of recurrent genes with any partner gene, by using an approach named "NGS-Target Capture". We developed two alternative protocols on RNA: Ovation Fusion Panel Target Enrichment System (Nugen, custom panel with 77 genes) and the TruSight RNA Pan-Cancer (Illumina, predesigned with 1385 genes). These approaches were set on MiSeq platform (Illumina). All data were analyzed by 'BreakingPoint' and tools, available on platform 'Illumina BaseSpace' Cloud, such as TopHat and RNA-Seq Alignment.

Results. Six well-characterized positive cases, with already known fusion genes (including p210 and p190 BCR/ABL1, ETV6/RUNX1, P2RY8/CRLF2, MLL/AF4) were transversally analyzed with the two protocols to compare the different library preparation flowcharts in terms of amount of RNA, timeline, man-effort, efficiency and cost. We analyzed a large cohort of 287 patients, selected as either High Risk for MRD or relapsed cases. Overall, using both methods and in particular N=88 and N=207 (8 in common) by Nugen and Pan-Cancer panels respectively, we detected N=144 (50%) fusion genes. Focusing our attention to next pediatric ALL protocol, named ALL-BFM-2017, which will start at the end of this year we analyzed a consecutive series of diagnoses selected by MRD at TP1(d33)$\geq 5x10^{-4}$, enrolled in 2017. By this strategy we identified a cohort of N=57 patients. So far, we analyzed 41/57 patients obtaining the following results: we identified N=7 fusions by conventional RT-PCR (such as t(9;22) and t(12;21) and N=12 by RNA Target Capture. In particular, these fusion genes were involving recurrent genes such as ETV6, EBF1, MLL, TCF3, ZNF384, PAX5and JAK2. At day +78, only 24/34 patients at final stratification were classified as HR (High Risk) or SER (Slower Early Responder) carrying 10 out of 12 fusions.

Conclusions. We conclude that RNA-target-NGS supported by purpose-built bioinformatics is the most promising and feasible to be introduced in diagnostic laboratory activity, being able to detect both conventional and novel fusion genes. These results provide important insights into the genetic basis of treatment failure in ALL and have implications for the early detection of mutations driving relapse.

CO008

PH-LIKE SIGNATURE IN CHILDREN WITH DOWN SYNDROME ALL

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Introduction: Children with Down Syndrome have a dramatic increased risk for ALL (DS-ALL), their third cause of death, due to both increased chemotherapy-related toxicity and intrinsic resistance to therapy, thus demanding the development of tailored therapeutic strategies. Cytogenetic abnormalities common in childhood ALL and contributing to risk based treatment assignment, i.e. t(12;21) and hyperdiploidy, are less frequent in DS-ALL, which is often characterized by alterations in CRLF2 gene, instead. Studies that identified poor-prognosis genetic groups such as Philadelphia Chromosome-Like (Ph-like) ALL usually excluded the DS-ALL patients from the analyses. Aim of the study was to retrospectively evaluate incidence and prognostic value of Ph-like signature in children with DS-ALL treated within previous AIEOP-BFM protocols.

Methods: We included 66 DS-ALL patients enrolled in the AIEOP-BFM ALL2000, ALL-R2006 and ALL2009 studies in Italian centers (AIEOP) from 2000 to 2014. Gene expression profiling was analyzed by Affymetrix HG-U133 Plus2.0 arrays; CRLF2 alterations were analyzed by RQ-PCR and FISH; digital MLPA-ALL (MRC-Holland, Amsterdam, the Netherlands) were performed to identify DNA copy number variations; RNA Next Generation Sequencing-Targeted Capture strategy (NGS-TC, MiSeq Illumina platform) for the detection of fusion genes.

Results: The majority of the AIEOP DS-ALL patients displayed a Ph-like ALL gene expression signature (31/47, 66.0%). Twenty-four out of 31 Ph-like patients were positive for CRLF2 alterations (15 P2RY8-CRLF2, 5 IgH@-CRLF2 and 4 CRLF2 overexpression), one carried a fusion involving ABL1 and one was positive for a translocation involving PAX5. Twelve Ph-like DS-ALL patients showed IKZF1 deletions (11/12 with co-occurring CRLF2 alterations) and 7 of these were "IKZF1plus" (IKZF1 deletions co-occurring with deletions in CDKN2A, CDKN2B, PAX5, or PAR1 in the absence of ERG deletion). They more frequently presented deletions in CDKN2A/B genes (30% vs. 13%). Interestingly, none of the Ph-like negative DS-ALL patients were positive for CRLF2 alterations or IKZF1 deletions, while they more frequently carried ETV6 deletions (43.8% vs. 19.4%). Ph-like DS-ALL patients had an inferior event-free survival (EFS) (49.7%±11.2 vs. 66.3%±15) and increased cumulative incidence of relapse (CIR) (40.5%±11.1 vs. 13.8%±21.2) compared to Ph-like negative patients. Updated results will be presented.

Noteworthy, the Ph-like positive patients were more frequently allo-

cated in the non-MRD High risk subgroup (8/31, 25.8% in the Standard risk and 17/31, 54.8% in the Intermediate risk).

Conclusions: The majority of the AIEOP DS-ALL patients displayed a Ph-like gene expression signature, a few of them with new class fusion genes. This profile has an impact on the outcome of DS-ALL and alternative targeted therapies could be planned, even considering the high toxicity for the multi-chemotherapy experienced by those patients.

Monoclonal Gammopathies and Multiple Myeloma 1

CO009

RARE, BUT COMPLEX CHROMOSOMAL REARRANGEMENTS, CAUSED BY SINGLE-STEP OR STEPWISE CATASTROPHIC GENOMIC EVENTS, SIGNIFICANTLY IMPACT ON MULTIPLE MYELOMA PATIENTS PROGNOSIS

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Background: Multiple Myeloma (MM) is a genetically complex disease, characterized by the recurrence of several chromosomal aberrations, which impair the disease prognosis. Besides these, the use of genome wide technologies has recently highlighted the existence of heterogeneous chaotic genomic events, generically defined “”Complex Catastrophic Events”“(CCEs), including chromothripsis (caused by single-step genomic events) and chromoanagenesis/stepwise CCEs (consequence of multiple, small and sequential genomic events, occurring throughout subsequent cell cycles). The prognostic impact of CCEs has not been yet fully elucidated. Aims of the present study were (1) to set up a reliable bioinformatic method, able to distinguish, characterize and dynamically define the different CCEs, as observed in the genomic landscape of MM patients (pts) and (2)to correlate their presence with the disease prognosis.

Patients And Methods: 473 newly diagnosed MM pts have been included in the present study. Genomic data have been obtained by SNPs arrays (Affymetrix 6.0 and CytoscanHD), as performed on BM-CD138+ enriched cell fractions; data were analyzed with ChAS v3.3 and Power Tools (Affymetrix), Rawcopy and personalized R scripts.

Results: An ad-hoc algorithm, able to both precisely identify and dissect CCEs, was set up and tested on genomic data of all pts. Criteria able to discriminate among the 2 different CCEs were defined, by taking into account both previously reported guidelines for CCEs identification and the MM-specific, highly heterogeneous genomic contest. Overall, 62 pts (13%) were shown to carry at least one CCEs: 43/62(69%) and 19/62(31%) carried either chromothripsis or progressive catastrophic events, respectively; both events were scattered across the whole genome, with any locus-specific bias. Pts with chromothripsis were more likely to carry both IgH translocations and chr17p del, whereas pts with progressive catastrophic events were mostly hyperdyploid and carried chr1q amp (Table 1).

Table 1.

EVENTs		Patients	t(4;14)	t(11;14)	t(14;20)	t(14;16)	t(6;14)	HD	Loss 17p (TP53)	Gain 1q	Loss 1p	Loss 13	ISS 3
Complex Catastrophic Events	Chromotripsis-like events	43 (9%)	14 (33%) *p=0.02	2 (5%)	3 (7%) *p=0.018	4 (9%)	/	22 (51%)	11 (26%) *p=0.002	20 (47%)	4 (9%)	26 (60%)	12 (28%)
	Step Wise-like events	19 (4%)	3 (16%)	1 (5%)	1 (5%)	1 (5%)	/	15 (79%) *p=0.018	3 (16%)	12 (63%) *p=0.014	3 (16%)	12 (63%)	5 (26%)
No Cathastrophic Events	Others MM	411 (87%)	71 (17%)	58 (14%)	3 (<1%)	16 (4%)	3 (<1%)	218 (53%)	36 (9%)	137 (33%)	47 (11%)	204 (50%)	108 (26%)

The onset of CCEs has been shown to impact on pts’ progression-free and overall survival (PFS, OS), with HR of 1.4652 (p=0.022) and 2.004 (p=0.001), respectively. In particular, progressive catastrophic events (both chromoanagenesis and stepwise) had a greater impact on PFS (HR 1.8382, p=0.024) and on OS (HR 2.89, p<0.001), as compared to chromothripsis events (PFS: HR 1.27 p=0.224; OS: HR 1.491 p=0.124).

Conclusions: The occurrence of genomic catastrophic events significantly impact on both OS and PFS of MM pts. Despite the co-segregation with low/intermediate-risk genomic aberrations, stepwise events seem to have a more adverse prognostic impact on survival, as compared

to chromothripsis. The use of genomic-wide technologies, coupled with specific bio-informatics tools, might help to more deeply dissect the role of these genomic events in myelomagenesis, as well as in the disease progression.

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CO010

TARGETING OF DEREGULATED LNCRNA NEAT1 AFFECTS MULTIPLE MYELOMA ACTIVITY

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Introduction: MM is a fatal malignant proliferation of antibody-secreting bone marrow PCs characterized by a marked genomic instability. The discovery of lncRNA has added a further layer of complexity to the pathobiology of the disease. NEAT1 is a lncRNA located at 11q13, transcribed in two different isoforms (3.7 Kb and 22.3 Kb), retained in the nucleus where it forms the core structural component of the paraspeckle sub-organelles. It may act as transcriptional regulator for numerous genes, including some involved in cancer progression.

Methods: lncRNA transcriptional profiles were generated on GeneChip® Human Gene 2.0 ST microarray in purified bone marrow PCs (>90%) from 50 MM, 15 PCL and 4 normal donors. qRT-PCR has been used to validate array data using two distinct couples of primers, able to amplify either both isoforms (mapped to 5' region) or the long variant of NEAT1 (3' region). We designed specific LNA-gapmeRs to silence NEAT1 in H929, AMO-1 and U266 MM cell lines by gimnotyc delivery; efficiency of silencing was evaluated by qRT-PCR and RNA-FISH. Proliferation and cell cycle were investigated by BrdU incorporation assay and FACS analysis, respectively. Protein expression was evaluated by Western Blot and the pH2A.X distribution was analyzed by IF technique. Luminometric assay was used to investigate cell viability, ROS production and caspase cleavage upon silencing.

Results: The transcriptional analysis identified NEAT1 upregulation in pathological samples compared to healthy controls. We did not observe any significant differences in expression levels either between different stages of the disease or molecular subgroups of MM cases. Gymnotic delivery of 5μM of in-house designed LNA-gapmeRs significantly reduced NEAT1 expression in all the HMCL tested when compared to their relative control. NEAT1 silencing was able to antagonize MM cell proliferation, as suggested by a significant decrease of the number of growing cells and the S-phase of the cell cycle. Furthermore, NEAT1 silencing was able to trigger apoptosis *in vitro* as revealed by the increase of Annexin+/7AAD+ cells and of cleaved PARP and Casp3 fractions. NEAT1 depletion in MM cells was also associated to increased intracellular ROS levels and induced DNA damage. Finally, we found that NEAT1 silencing had a synergistic anti-proliferative effect in MM cells when combined with proteasome inhibitors, and was additionally able to overcome bortezomib resistance *in vitro*. So far, preliminary data are indicating anti-proliferative effect of NEAT1 silencing *in vivo* in xenograft mouse model.

Conclusions: In our study, we investigated the involvement and the possible pathogenetic role of NEAT1 in MM. We provided novel important insights concerning the NEAT1-dependent regulation of MM proliferation and preliminary data that support a LNA-gapmeR NEAT1-targeting strategy as new potential powerful therapeutic agent for MM treatment.

CO011

THE LONG NON-CODING RNA ST3GAL6-AS1 DEREGULATED IN MULTIPLE MYELOMA

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Introduction: During the last two decades, long non-coding RNAs (lncRNAs) relevance has been highlighted by a large number of studies. LncRNAs are non-protein-coding transcripts, longer than 200 nucleotides, which can act to regulate gene expression and be involved in cancer. More recently, some groups evidenced the impact of deregulated lncRNAs in Multiple Myeloma (MM). In previous report, we demonstrated that deregulated patterns of lncRNAs expression are associated with distinct MM molecular subtypes (Ronchetti *et al.*, Oncotarget, 2016).

Aims: Characterizing the RNA structure and explore the functional role in MM human cell lines (HMCLs) of ST3GAL6-AS1, the unique up-modulated lncRNA in MM patients compared with healthy donors.

Methods: A custom annotation pipeline was used to investigate lncRNA profiles on GeneChip® Human Gene 2.0 ST microarray in highly purified bone marrow PCs from 50 MM primary tumors and 4 normal donors. Real-time PCR (QT-PCR) was performed to confirm array data. Predicted splicing of ST3GAL6-AS1 transcripts were examined by qualitative PCR and Sanger sequencing in HMCLs and primary tumors. lncRNAs subcellular localization was evaluated by fractionated HMCLs nuclear and cytoplasmic RNA. HMCLs were treated with actinomycin D to determine the half-life of the lncRNAs. siRNA silencing of ST3GAL6 on HMCLs was performed by Neon Transfection system.

Results: We analyzed the long non-coding RNA fraction of the transcriptome of 50 MM patients using arrays that investigate more than unique 10000 sequences. Importantly, we found ST3GAL6-AS1 as the unique significant overexpress lncRNA in MM samples compared to healthy donors. ST3GAL6-AS1 maps to 3q12.1 and is antisense to ST3GAL6, a protein involved in homing and *in vivo* engraftment of HMCLs and correlated with shorter overall survival in MM patients (Glavey *et al.*, Blood, 2014). The validation of array data by QT-PCR confirmed the overexpression of ST3GAL6-AS1 in MM sample. Analysis on HMCLs revealed that ST3GAL6-AS1 was overexpressed and equally localised in nuclear and cytoplasmic fractions. Furthermore, molecular analysis of the lncRNA in HMCLs and primary tumors showed the presence of a polymorphic splicing nucleotide variant (rs13065271) with the retention of a 128bp intron in the transcript. In homozygous mutated HMCLs we observed a prevalent nuclear localization of ST3GAL6-AS1, as well as a lower expression and reduced half-life of its transcripts. Moreover, ST3GAL6-AS1 and ST3GAL6 displayed a significant correlation in their expression levels. siRNA silencing of ST3GAL6 in HMCLs caused down-regulation of ST3GAL6-AS1, suggesting a possible co-regulation mechanism.

Conclusions: Our data indicate that ST3GAL6-AS1 is significantly deregulated in MM patients. Furthermore, the occurrence of a polymorphic variant leading to an alternative splicing in ST3GAL6-AS1 may have potential relevance in the transcript stability and its functional role.

CO012

TLR4 SIGNALING PROMOTE MESENCHYMAL STEM CELLS (MSC) COMMITMENT TO PROMOTE TUMOR MICROENVIRONMENT TRANSFORMATION IN MULTIPLE MYELOMA

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Introduction: MSC, through a complex crosstalk with neighboring cells/factors, can inhibit many effector functions of immune cells, thereby promoting an immunosuppressive state in the tumor microenvironment. Recently, we demonstrated *in vitro* the key role played by MSC in multiple myeloma (MM) bone marrow, making it an immune-tolerant milieu by favoring immunosuppressive abilities of surrounding myeloid cells. Since it has been demonstrated a connection between the stimulation of specific Toll-like receptors (TLR) and MSC activation status, including two distinct phenotypes defined MSC1 (TLR4-dependent) or MSC2 (TLR3-dependent), we hypothesize that MM-MSC have an inflammatory phenotype associated to TLR signaling.

Results: Using specific agonists for TLR4 (LPS) or TLR3 (poly(I:C)), we observed that healthy MSC acquired the same immunological alteration of SMM- and MM-MSC after a pre-treatment with LPS. Moreover, wester blotting analysis confirmed the activation of TLR4/MyD88 pathway in MM-MSC but not in HC-MSC. To examine if myeloma plasma cells (PC) play a role in MSC polarization, before performing co-cultures with PBMC, we pre-treated HC-MSC with MM cell lines. Plasmacell pre-treatment drove healthy MSC to activate neutrophils in immunosuppressive and pro-angiogenic cells. Therefore, we investigated if PC activated TLR4 pathway in healthy MSC and we found that co-culture with PC induced IRF3 nuclear translocation, indicating the involvement of a TLR4-MyD88-independent pathway in MSC commitment. Next, we explored the effects of the "activated" status of MM-MSC investigating their pro-tumor role *in vivo*. Six days after implanting a mixtures of fluorescently labeled MM cells plus HC- or MM-MSC, zebrafish co-injected with PC and MM-MSC showed enhanced tumor colonization and growth (calculated as tumor volume and fluorescence intensity) compared with animals injected with PC and HC-MSC (control) ($p<0.05$). Flow cytometry detection of hCD138+ cells confirmed less MM cells in zebrafish injected with PC and HC-MSC ($p<0.001$). Therefore, we analyzed the expression of the master regulator transcription factors for Th1/Th2 (tbx21 and gata3) and Th1- and Th2-type cytokines to better assess *in vivo* the involvement of the immune escape mechanisms promoted by co-injection of PC with MM-MSC. As compared to control animals, gata3, IL-4 and IL-13 were significantly upregulated in zebrafish injected with PC plus MM-MSC, revealing that MM-MSC and PC mixture promoted a Th2 responce. To investigate TLR4 role, we used TAK-242 to inhibit the signaling in MM-MSC before injection in zebrafish. Animals co-injected with PC and MM-MSC pretreated with TAK-242 showed 48% less tumor engraftment compared to zebrafish injected with PC and MM-MSC.

Conclusions: TLR4 signaling plays a pivotal role in MSC commitment towards an inflammatory phenotype which is associated with a tumor permissive microenvironment.

CO013

TRYPTOPHAN SHORTAGE DUE TO IDO-1 EXPRESSED BY HIGH-DENSITY NEUTROPHILS INDUCE IMMUNE-SUPPRESSION AND AN ADAPTIVE RESPONSE IN PLASMA CELLS OF MULTIPLE MYELOMA

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Background: There is an increasing interest about the role of amino acid degrading enzymes in cancer immunotherapy. In multiple myeloma (MM), several groups including ours showed that immune-suppression due to aminoacid starvation is clinically relevant, thus we investigated if tryptophan shortage could hamper immune function in the progression from MGUS through MM.

Materials and Methods: We first measured the amount of tryptophan and its degrading enzyme 2,3-indoleamine deoxygenase (IDO-1) in sera obtained from bone marrow and peripheral blood of 15 MGUS, 10 smoldering MM (sMM), 15 newly diagnosed and 10 relapsed MM. Second, we evaluated the main cellular source of circulating IDO by western blot and immune fluorescence of neutrophils, monocytes and neoplastic plasma cells. Third, we explored if the immune-suppressive activity of MM-LDN could be recovered by treatment in-vitro with 200 nM epacadostat, an IDO-1 inhibitor currently under investigation in phase I-II trials of immunotherapy in solid cancers. Fourth, we explored if tryptophan shortage could induce an adaptive response to MM cells MM1.s, OPM2 and U266 *in vitro* and mediate refractoriness to bortezomib, melphalan and lenalidomide.

Results: IDO-1 was increased in both bone marrow and peripheral blood of MM patients compared to MGUS and healthy subjects ($p=0.002$). Conversely, tryptophan was reduced (more in peripheral blood than in bone marrow) in MM versus MGUS patients and kynurenine (a product of tryptophan degradation) increased ($p=0.001$). T- cell function, evaluated as expression of HLA-DR and CFSE expression upon stimulation with 5ng/mL phytohemaglutinin (PHA) for 72 hours, was hampered by co-colture at ratio 1:4 with MM-derived neutrophils, and only partially reverted by treatment with 200 nM epacadostat. Surprisingly, increased concentration up to 1uM (achieved *in vivo* in published studies on solid cancers) of epacadostat disclosed a dose-dependent toxicity against T-cells, more evident for CD4+ than CD8+ subpopulations, limiting further use *in vitro*. MM cells expressed IDO-1 but their viability was not affected by exposure to epacadostat up to 72 hours. Tryptophan shortage (1000-10nM) did not affect cell proliferation and cell cycle of MM cell lines tested either, while induced T-cell apoptosis within 48 hours. In two human myeloma cell lines MM1.s and U266, progressive tryptophan shortage induced an adaptive response through increased expression, time and dose-dependent, of ATF4-ASNS-CHOP-GADD34, part of GCN2 signaling. *In vitro,* sub-toxic treatment with 5nM bortezomib, 10uM lenalidomide or 10uM melphalan for 24 hours showed synergic effect only between melphalan and epacadostat.

Conclusions: IDO-1 increase and tryptophan shortage are associated to MM progression. Neutrophils are IDO-1 positive and mediate immune-suppression that can be reverted only partially by treatment with 200nM epacadostat.

CO014

MIR-21 SUPPRESSION IMPAIRS TH17 RELATED BONE DISEASE IN MULTIPLE MYELOMA

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Introduction: Bone disease (BD) is a hallmark of MM and several findings indicated that IL-17 producing CD4+ T cells (Th17) play a central role in triggering MM-BD. IL-17 levels are increased in MM bone marrow microenvironment (BMM), favoring OCL-bone damage. MicroRNA-21 (miR-21) is a pro-inflammatory and oncogenic miRNA, that drives MM cell proliferation and promotes RANKL expression by BM stromal cells (BMSCs). Interestingly, miR-21 promotes Th17 differentiation and function. Based on these premises, in this work we asked whether miR-21 may have a role in Th17 mediated BD.

Methods. Th17 cells were freshly isolated from MM/normal donor (ND) BM. *In vitro*, ND-naive CD4+ T cells, exposed to Th17 cytokines, became consistently CCR6+CD161+CD4+ and expressed IL-17, IL-22 and RORC (Th17 signature). miR-21 inhibitors (miR-21i) and scramble miRNAs (SC) were transfected by Neon® Transfection System at 100nM. OCLs derived lacunae on dentin slices with/without Th17 were measured by NIH ImageJ software. Digested SC-/miR-21i-Th17 proteins were labeled by tandem mass tags for whole-proteome analysis and phosphopeptide enrichment. Pathway analysis was conducted by Ingenuity Pathway Analysis (IPA, Qiagen). Comparisons between groups were made with student's t-test (Graphpad Prism version 6.0).

Results. We detected miR-21 levels within MM BM Th17. miR-21 turned to be higher in MM as compared to controls. MM with osteolytic lesions (OLs) showed significantly higher miR-21 levels as compared to MM without OLs (Figure 1). MM-Th17 expressed significantly higher

RANKL as compared to controls both at surface and mRNA level. To assess miR-21 activity, Th17 derived from naive CD4+ T cells *in vitro* were exposed to miR-21i or SC. MiR-21i impaired Th17 differentiation by reducing IL-17, IL-22 and RORC. miR-21i abrogated Th17-dentin pits formation by OCLs. We applied quantitative phosphoproteomics analysis and interrogated IPA software to assess miR-21 dependent protein interactions. This approach allowed to identify several miR-21 direct/indirect target proteins and the related pathways. We found 1134 phosphorylated proteins with 386 significant changes as compared to controls, that were mapped into 7 networks. Among them, six phosphoproteins were miR-21 specific targets. IPA analysis disclosed RANKL among the top upstream proteins. Overall, the main miR-21 regulated axis led to STAT3 activation and RORC expression. miR-21i impaired this axis by up regulation of the protein inhibitor of activated STAT3 (PIAS3), that abrogated STAT3 activity, and the inhibition of PI3K/AKT and 14-3-3 pathways, that correlated with increased levels of FOXO1 and PDCD4 proteins.

Conclusions. These data showed that miR-21 antagonism negatively regulates Th17- differentiation and OCL bone resorption. MiR-21 targeting may represent an attractive innovative strategy to reset the pro neoplastic BMM, that supports malignant PC survival, proliferation and triggers BD.

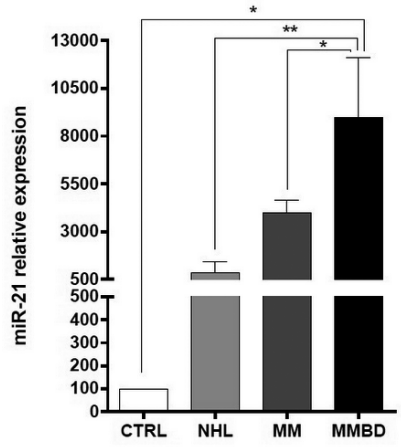

Figure 1.

CO015

IMPLEMENTATION OF AN EVO-SCORE IN A WHOLE-GENOME ANALYSIS REVEALS GENETICALLY DISTINCT MULTIPLE MYELOMAS WITH OPPOSITE EVOLUTIONARY TRAJECTORIES

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Background: Multiple Myeloma (MM) disease progression is often coupled with radical changes of the genomic sub-clonal architecture, which in turn might impact the tumor-specific aggressiveness and invasiveness over time. Therefore, MM patients (pts) are unlikely to maintain sustained MRD negativity status and the disease typically recurs. Copy Number Alterations (CNAs) are the most frequent alterations in MM, acting both as evolutionary and prognostic markers: indeed, gene-specific CN changes over time potentially hold key information on the functional mechanisms of tumor clones resistance. Aim of the study was to design a bioinformatic analysis pipeline measuring, for each pts, at gene level, the "extent of evolution", thus defining an EVO-score, able to resume the gene-specific evolution, as induced by any specific therapeutic selective pressure.

Patients and Methods: 63 MM pts, up-front treated with bortezomib-based regimens (mainly VTD), were included in this study. SNPs array (6.0 and CytoscanHD, Affymetrix) analyses were performed in BM-CD138+ cell fractions, as collected from pts both at diagnosis and at relapse. Data were analyzed with dedicated R packages and custom scripts.

Results: In order to dissect and deeply characterize the selective pressure acting on MM patients' genome evolution, we developed an analysis algorithm able to assess the CNAs changes between two disease phases of any functional genomic region (i.e. coding genes and miRNA). Results obtained by analyzing the whole cohort of MM pts were next bi-clustered, by employing three linkage-methods, hence revealing common subgroups of genes and pts showing similar behaviors, under the same therapeutic selective pressure. Figure 1 depicts the two main evolution trajectories highlighted, i.e. branching (B, 37 pts) and stable (S, 26 pts). At diagnosis, chr 19 CN gains, LOH events and chr 1q CN gains significantly recur in the B-pts' genomic landscape (p=.005, .008 and .052, respectively); in contrast, chr 8q CN losses were exclusively observed in the S-pts' genomic background (p=.001). To get deeper insight into critical pathways linked to the process of clonal evolution under VTD selective pressure, an EVO-score was assigned to each gene affected by a CN changes for those pts with branching trajectory, in order to define the "evolution amount" acting on every single genetic locus. The top-1000 most evolving genes were then subjected to a Gene Set Enrichment Analysis, thus revealing that the "osteoclast differentiation" pathway was significantly involved in the evolution process (p<0.001, Benjamini adj.).

Conclusions: To both estimate and define the whole-genome CNAs evolution under VTD selective pressure, an EVO-score has been assigned to each gene of each pts, thus both describing two main, genomically distinct, evolution trajectories and highlighting the cellular pathway more significantly involved in the evolution processes.

Acknowledgements: AIRC (MC), Fondazione Berlucchi (CT)

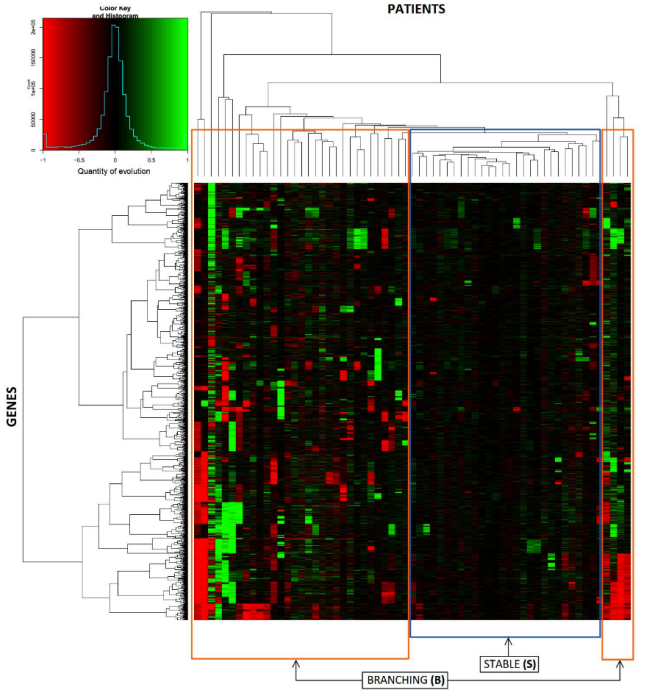

Figure 1.

CO016

SPLICING FACTOR 3B SUBUNIT 1 (SF3B1) REPRESENTS A NOVEL DRUGGABLE TARGET FOR MULTIPLE MYELOMA PATIENTS

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Introduction: Multiple Myeloma (MM) is a genetically complex and heterogeneous hematological neoplasm in which several survival pathways, including DNA-damage, are often deregulated. Despite novel therapies have remarkably improved MM patients outcome, drug resistance develops, partially due to the protective role of the bone marrow (BM) microenvironment. Emerging data suggest a crucial role for RNA splicing deregulation in hematologic malignancies such as MDS and CLL. In such a scenario, biological role of spliceosome deregulation in MM remains to be elucidated. Here, to characterize the role of such abnormality, we assessed therapeutic relevance of spliceosome core component SF3B1-targeting using preclinical MM models.

Methods: A comparative gene expression analysis (GEP), focused on established splicing signature, was performed by using publically available datasets. Next, SF3B1 expression analysis was performed on a panel of MM cell lines as well as in CD138+ tumor cells derived from MM patients; PBMCs from healthy donors (HDs) were used as negative control. The cytotoxic effect of SF3B1 chemical inhibitor Meayamycin B was measured using MTT-based assay and Annexin-V/PI staining. Mechanistic studies were performed with western-blotting and immunofluorescence analyses. The effect of Meayamycin B in presence of BM microenvironment (using IL6/IGF1 addition or MM patients-derived BM stromal cells co-coltured with Nanoluciferase-expressing MM cell lines) was investigated. To further support our findings, different genetic approaches (shRNAs and siRNAs) specifically targeting SF3B1 were adopted.

Results: GEP analysis showed deregulated expression of genes encoding spliceosome related-proteins in MM compared with MGUS or HDs, supporting the pivotal role played by such machinery in the biology of such tumor. Indeed, we found higher SF3B1 expression in MM cell lines and primary cells compared to HD PBMCs. Of note, SF3B1 expression was further increased in presence of BM-mileu as shown in MM cell lines co-coltured with patients-derived BMSC. Next, we examined the therapeutic relevance of SF3B1 targeting in MM by testing a specific chemical inhibitor or its depletion. Meayamycin B showed a potent anti-tumor effect on both human cell lines as well as primary cells with IC50 values ranging between 0.5 and 3 nM. Co-colture experiments showed that such activity was not abolished in the presence of BM microenvironment. Indeed, SF3B1 depletion with RNAi approaches led to massive apoptotic cell death. Mechanistic studies revealed huge genomic instability following SF3B1 targeting with an impaired DNA-damage repair mechanisms efficiency, as highlighted by increased $^{\gamma}$H2AX foci formation and RAD51 reduced expression.

Conclusions: Overall our data show that inhibition of SF3B1 impairs MM cell growth and survival. These results support a role of spliceosome deregulation in MM pathogenesis providing also the rationale for novel therapies targeting spliceosome-core elements to improve MM patients outcome.

Chronic Lymphocytic Leukemia and Chronic Lymphoproliferative Disorders

CO017

MIR-146B DOWN-REGULATION IS RESPONSIBLE FOR FAS LIGAND-MEDIATED NEUTROPENIA IN T-LARGE GRANULAR LYMPHOCYTE LEUKEMIA

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Introduction: T-Large Granular Lymphocytes (T-LGLs) leukemia (T-LGLL) is a chronic lymphoproliferative disorder characterized by the clonal expansion of T-LGLs. The most frequent clinical feature observed in T-LGLL patients is neutropenia. Mechanisms leading to its development are not completely established, although literature data provided evidence of the involvement of soluble Fas Ligand (FasL) in this process. Consistently, we demonstrated that neutropenic patients were characterized by higher levels of FasL than non neutropenic ones. However, the mechanism through which FasL production is regulated still remains unknown. Since many microRNAs (miRNAs) are regarded as important gene expression regulators, often involved in the pathogenesis of cancer, the aim of this study was to investigate whether the increased FasL production in neutropenic patients was due to an altered expression of miRNAs.

Methods: T-LGLs were purified by FACSAria cell sorter from PBMCs of untreated T-LGLL patients. High throughput and single miRNA analysis were carried out on purified LGLs by using the TaqMan® Human microRNA Array and Assays, respectively. Transfection with miR-146b mimic was performed using the Amaxa Nucleofactor and the Ingenio Electroporation Solution. Transcriptional and protein expression levels were evaluated by Real Time-PCR and Western Blot (WB) assays.

Results: We assessed the expression of 756 mature miRNAs on purified T-LGLs. miRNA differentially expressed between patients characterized by neutropenia vs. those with normal absolute neutrophil count (ANC) were analysed for correlation with ANC. miR-146b expression, found down-regulated in neutropenic patients, was the only one correlated with ANC. To investigate miR-146b potential role in neutropenia development, we transfected purified T-LGLs with a miR-146b mimic. Our data showed that restoration of miR-146b led to a decrease of FasL mRNA, without changes in FasL primary transcript, compared to control. These data indicated that miR-146b affected FasL expression at a post-transcriptional level. Since FasL was not identified among the putative miR-146b target genes, it is suggested that miR-146b could indirectly regulate FasL expression. Therefore, we checked for genes involved in mRNA stability and we found that miR-146b down-regulation was responsible for increased mRNA levels of Human Antigen R (HuR). HuR is a known mRNA stabilizer reported to be required for FasL expression in T-lymphocytes. Consistently, by WB assays we demonstrated that in T-LGLs of neutropenic patients HuR endogenous protein levels were higher than in T-LGLs of non neutropenic ones. HuR-mediated FasL mRNA stabilization explains the increased FasL expression observed in neutropenic patients.

Conclusions: In this work we suggest a pathogenetic link between a defective miR-146b expression and FasL-mediated neutropenia in T-LGLL, providing new potential therapeutic targets for neutropenic T-LGLL patients treatment.

CO018

TP53 CLONAL AND SUBCLONAL ARCHITECTURE IN CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS UNDER IMMUNOCHEMOTHERAPY AND IBRUTINIB

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Introduction: In chronic lymphocytic leukemia (CLL), analysis of TP53 defects is mandatory prior to treatment. We analyzed the TP53 clonal and subclonal architecture in CLL patients (pts) under multiple lines of chemoimmunotherapy (CIT) and ibrutinib (IBR).

Methods: Forty pts (Table 1) underwent a longitudinal TP53 monitoring (120 samples) by Sanger sequencing (SS) before each line of CIT; 14 also received IBR after a median of 1.5 CIT lines (range: 1-4) (IBR exposition: 2.1 to 4 years in 12/14 pts). Overall, 24/40 pts (60%) had at least 3 time points. Samples were analyzed by ultra-deep sequencing (UDS) on a MiSeq sequencer (Illumina, Inc.) (5000X coverage/base). Variants were checked on the IARC TP53 database. The variant allele frequency (VAF) was corrected to cancer cell fraction (CCF) by the % of CD19+/CD5+ cells.

Table 1.

Clinical and biological characteristics of 40 patients.	
Gender	29 M (72.5%) / 11 F (27.5%)
Median age (range)	54.7 years (36-74)
Median follow-up from diagnosis	9.9 years (3.5-22.2)
Median time from diagnosis to clonal *TP53* mut	9.9 years (2.7-21.3)
Germline *IGHV*	29/39 (74.4%)
del17p	10/37 (27%)
Type of *TP53* mutations	61
	43/61 (70.5%): missense
	11/61 (18%): indels
	5/61 (8%): splicing
	2/61 (3.5%): nonsense
Mean lymphocyte count (x10^9/L):	
At first time point (n= 40 pts)	40.6 (1-161.8)
Pre-ibrutinib (n=14 pts)	48.5 (1.5-137.8)
Post-ibrutinib (n=14 pts)	15.3 (1.4-100)

Results: By SS, 23/40 pts carried a clonal TP53 mutation (TP53-mut). By UDS, 28/40 pts harbored from 1 to 11 TP53-mut, for a total of 151 mutations. Forty-four of the 151 mutations (29.1%) were clonal (mean VAF: 31.4%; 10.3%-80.8%) and 107/151 (70.9%) were subclonal (mean VAF: 2.93%; 0.46%-9.89%, with a VAF≤5% in 94/107). Pts followed 5 patterns: 1) clonal TP53-mut present from the first time point (n=3); 2) subclonal TP53-mut evolving into clonal +/- additional minor subclones (n=10); 3) clonal TP53-mut emerging with additional subclones not predicted by a previous subclone (n=10); 4) subclonal TP53-mut which never evolved to clonal (n=5); 5) absence of any detectable mutation in all time points (n=12). In group 2, the mean TP53-mut CCF increased from 5% to 43.5% (p<0.0001). In 23 pts with available pre-treatment samples (n=30), 6 mutations (4 subclonal/2 clonal) were detected, whilst the post-treatment samples (n=64) showed 86 mutations (56 subclonal/30 clonal) (p<0.0001). The mean CCF was 5.1% (0.9%-17.2%) vs 14.2% (0.9%-80.7%) (p=0.007), respectively. Furthermore, we followed the 14 pts who received IBR. During the CIT phase (mean duration: 4 years), 14 novel mutations developed, 3 subclonal evolved to clonal, 13 persisted and none was lost. Contrariwise, during the IBR phase (mean duration: 2.5 years), only 2 subclonal novel mutations emerged, one evolved to clonal, 5 clonal decreased to subclonal, 16 (4 clonal/12 subclonal) persisted and 10 (1 clonal/9 subclonal) were lost (p<0.0001). The mean CCF of the existing mutations remained stable before and after IBR treatment (20.7% vs 19.6%; p=NS), although the lymphocytosis significantly decreased (48.5×10^9/L vs 15.3×10^9/L; p=0.015).

Conclusions: Clonal TP53-mut emerging under CIT can be anticipated in at least half of the cases by subclones carrying the same mutation, according to the positive selection of resistant clones. Not all subclonal mutations have the same role: some evolve into major clones, others remain stable over time or disappear. IBR might decrease TP53-mut numerosity and complexity; however, the dominant clones may survive and give rise to subsequent relapses.

CO019

IBRUTINIB AFFECTS THE IMMUNE RESPONSE OF MACROPHAGES AGAINST ASPERGILLUS IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction: Chronic lymphocytic leukemia (CLL) is characterized by clinical complications related to alterations in the immune system. CLL cells escape immunosurveillance and model the surrounding microenvironment. Nurse-like cells (NLCs) differentiate from peripheral blood-derived monocytes in vitro and also are detected in lymph nodes of CLL patients. NLCs share several features with tumor-associated macrophages. Ibrutinib is an irreversible inhibitor of Bruton tyrosin kinase (Btk) able to target CLL cells. Btk is also involved in macrophage polarization. Our results have shown that ibrutinib targets NLCs impairing ability to polarize into M1 (inflammatory) macrophages, instead showing enhanced induction of M2-associated markers.

Methods: NLCs were generated in complete medium: cell surface markers and functional studies were performed after 24 hour of treatment with ibrutinib. Microarray-based gene expression profiles of NLCs treated or not with ibrutinib were evaluated after 24 hours and data were confirmed by real time PCR. Anti-aspergillus fumigatus activity of NLCs was analyzed by measuring the NLCs-induced hyphal damage by a (2,3)-bis-(2-methoxy-4-nitro-5-sulfenyl)-(2H)-tetrazolium-5-carboxanilide (XTT)-based colorimetric assay.

Results: Ibrutinib targeted and reduced the level of phosphorylated BTK in NLCs. We analyzed the effects of this inhibition on the macrophage component in CLL. Analysis of gene expression profiling showed that treatment with ibrutinib modified NLCs gene expression profile. The supervised analysis identified 566 differentially expressed genes. Among down-regulated genes, the most represented GO categories were related to immune system process, inflammatory response, immune response, cytokine activity, implying the ability of ibrutinib to modify the expression of genes implicated in immune function of NLCs. The down-regulated profile included several genes belonging to tumor necrosis factor receptor family and interleukin 1. We investigated the level of TNF-α and IL-1 production after treatment with ibrutinib either in presence or absence of Aspergillus fumigatus stimulation. We found a decreased expression of TNF-α and IL-1 after treatment with ibrutinib, on the contrary stimulation with Aspergillus conidia significantly stimulated its production that was counteracted by the presence of ibrutinib. Moreover, NLCs were able to induce a direct damage to the hyphae of Aspergillus fumigatus, that was significantly reduced by the treatment with ibrutinib.

Conclusions: Collectively, these data indicate that treatment with ibrutinib deeply modifies the macrophage population in CLL. These new insights may help to explain how ibrutinib allows invasive pulmonary aspergillosis by suppressing innate immune responses against Aspergillus.

CO020

IRF4 L116R MUTATION PROMOTES PROLIFERATION OF CHRONIC LYMPHOCYTIC LEUKEMIA B-CELLS BY INDUCING MYC

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Introduction: Interferon regulatory factor 4 (IRF4) is a transcriptional regulator of B-cell development and proliferation. IRF4 can play different and opposing roles according to the level of expression. Recently genome-wide association and whole-exome sequencing studies identified the IRF4 gene as associated with chronic lymphocytic leukemia (CLL) development. Single nucleotide polymorphisms in the IRF4 gene were associated with an increased risk of developing the disease. A first report identified the presence of a specific heterozygous missense somatic substitution (L116R) in IRF4 gene (1.3% frequency) located in the DNA-binding domain of the gene that was associated with higher IRF4 expression levels. IRF4 gene mutation (1.33%) is also identified among novel independent prognostic drivers in CLL for shorter time to first treatment.

Methods: We screened a multicenter cohort of 460 CLL patients at relevant clinical stages of disease with ARMS-PCR, with a mutation (L116R) specific primer. Then we transfected CD19+ CLL cells with IRF4-wt (wild type) or mutant plasmid vector. Transfected cells were analyzed for IRF4 expression levels by real-time PCR and for viability by Annexin/PI staining. We measured proliferation rate through different assays including Ki-67 and CFSE staining by flow cytometry and the incorporation of BrdU by colorimetric assay. Intracellular signaling pathways activating nuclear c-Myc were evaluated in IRF4 transfected CLL cells by immunoblotting.

Results: Two L116R IRF4 mutated patients were found in the cohort of relapsed/refractory (3.9% frequency, 2/51), one at the diagnosis (0.3%, 1/365) and one mutant Richter patient (2.3%, 1/44) through ARMS-PCR and Sanger sequencing. To analyze biological consequences of IRF4 mutation, we transfected CLL cells from patients with IRF4-wt or mutant plasmid vector and we cultured transfected cells on a feeder layer of 3T3-CD40L murine stromal cells. IRF4 expression level increased in transfected cells with mutant plasmid compared to IRF4-wt transfected cells, and spontaneous apoptosis was not found to be different. In our model, proliferation rate increased in IRF4 mutant CLL cells compared to IRF4-wt transfected cells, measured through Ki-67 (1.7 fold increase) and CFSE (2.9 fold increase) staining. Results were confirmed also in CLL cells transfected with mutant plasmid and stimulated with soluble CD40L/IL4+CpG/IL2, to activate cell division measuring BrdU incorporation (1.2 fold increase), compared to IRF4-wt cells. Interestingly c-Myc and pGSK3β/tGSK3β protein level increased in IRF4 mutant CLL cells as compared to IRF4-wt cells.

Conclusions: We found that IRF4 mutant CLL cells are more prone to proliferate than IRF4-wt cells. Collectively, our results indicate that IRF4 L116R mutation in CLL cells may be functionally active conferring to leukemic cells a proliferative advantage. This property of IRF4 mutated CLL may explain the adverse impact on disease progression seen in patients harboring IRF4 L116R mutation.

CO021

DISSECTING AND TARGETING TOLL-LIKE RECEPTORS SIGNALING IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction: Toll like receptors (TLR) are master regulators of innate immunity while at the same time they co-stimulate immune signaling in B-lymphocytes. TLR are also expressed in B-cell lymphoma cells, including chronic lymphocytic leukemia (CLL). CLL cells depend on external stimuli for survival and proliferation, and both antigen receptor and TLR are involved. In particular, the TLR9 ligand CpG can modulate cell viability, proliferation, apoptosis and chemoresistance *in vitro*. TLR9 stimulation can also induce NFKBIZ expression and IgM secretion thus regulating a central signaling route in leukemic cells pathobiology. We herein aimed at dissecting and targeting TLR-mediated signaling pathways in CLL. To inhibit TLR activation, we targeted IRAK4, the most upstream mediator of TLR signaling complex; specifically, we used small molecule drugs in different preclinical models of CLL including cell lines and primary leukemic cells.

Methods: Leukemic cell lines and primary malignant B-cells isolated from the peripheral blood of CLL patients (n=44 cases) were cultured *in vitro* with or without IRAK4 inhibitor in the presence or absence of the TLR9 ligand CpG. In addition, IRAK4 inhibitor was combined with fludarabine or BTK inhibitor to calculate the Combination Index. Cells were analyzed 48 hours after treatment for viability and apoptosis. Proteins and RNA were analyzed after 4-24 hours by Western blot and Real-time PCR analysis to monitor distinct signaling molecules and apoptosis regulators.

Results: IRAK4 inhibitor induced cell death in a dose dependent manner in both primary leukemic cells and cell lines; in detail, at a concentration of 10 microM, leukemic cells viability decreased to a mean of 40% as compared to untreated cells. The monocytic cell line Thp-1 used as internal control was not significantly affected by the treatment (cell viability >90%), while both Mec-1 and Mec-2 CLL cell lines showed an increased cell death after IRAK4 inhibition (cell viability <50%). Administration of CpG caused heterogeneous responses as previously observed by different groups including ours; regardless, IRAK4 inhibitor blocked the protective effect of CpG on cell viability. Co-administration of increasing doses of IRAK4 inhibitor and fludarabine exerted a synergic effect in both Mec-1 and primary leukemic cells (n=13), as measured by the Combination Index (CI<1). Combination of BTK and IRAK4 inhibitors at higher doses synergized in primary CLL cells (n=9). To note, TLR-induced NFKBIZ expression decreased when cells were treated with IRAK4 inhibitor in both cell lines and primary CLL cells, thus representing a specific biomarker of drug response. Moreover, distinct apoptosis regulators and MAP kinases were monitored after IRAK4 targeting.

Conclusions: These data may open novel potential therapeutic perspectives for the use of TLR signaling inhibitors in CLL, either alone or in combination with currently used drugs.

CO022

IN CHRONIC LYMPHOCYTIC LEUKAEMIA WITH COMPLEX KARYOTYPE, MAJOR STRUCTURAL ABNORMALITIES IDENTIFY A SUBSET OF PATIENTS WITH INFERIOR OUTCOME AND DISTINCT BIOLOGIC CHARACTERISTICS

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Introduction: In CLL the complex karyotype (CK) is a negative prognostic factor associated with an inferior outcome and a worse response to treatment, including novel agents. The CK is a heterogeneous cytogenetic category, including numerical and structural abnormalities. We investigated whether in CLL with CK the presence of numerical or structural chromosomal abnormalities could be associated with distinct clinical or biologic features.

Methods: 90 untreated CLL patients with CK diagnosed and treated between 2000 and 2017 according to NCI criteria were included. CK was defined in the presence of ≥3 clonal aberrations. The prognostic relevance of monosomies, trisomies, deletions, balanced translocations, unbalanced rearrangements and ≥5 abnormalities was then analysed and correlated to clinical and biological parameters including IGHV mutational status and mutations affecting NOTCH1, SF3B1, BIRC3 and TP53 genes. We then analysed the mRNA expression profiles of 23 patients with (n=11) and without (n=12) unbalanced rearrangements.

Results: The median age was 67.4 years. The median follow-up was 51.3 months. In multivariate analysis, age >65 years (p=0.038) and unbalanced rearrangements (p=0.025) were associated with a worse overall survival. Patients with unbalanced rearrangements had a lower incidence of 11q deletion (p=0.029) and trisomies (p=0.006) and a higher incidence of TP53 aberrations (p=0.014), monosomies (p=0.004) and a karyotype with ≥5 abnormalities (p=0.003). When considering time to first treatment, in multivariate analysis, advanced stage (p=0.001), unmutated IGHV (p=0.034) and unbalanced rearrangements (p=0.043) were associated with a negative prognostic impact. The analysis of mRNA expression profiles identified 160 differentially expressed genes (p <0.1, fold change cut-off >2.0). Using a combined set of genes, a clear-cut separation of the analysed samples was obtained (Figure 1). The presence of unbalanced rearrangements was associated with a deregulation of genes involved in cell cycle control and DNA damage response. Among these, TRPM4, RASGRF1, CTTBP2 and SLAMF1 may be of interest as they may have possible prognostic and therapeutic implications.

Conclusions: We have shown that CLL patients with unbalanced rearrangements may represent a subset of very high-risk CLL patients with distinct clinical and biologic characteristics. These patients should to be identified at the time of treatment and could be considered upfront for alternative treatments, including combinations of novel agents.

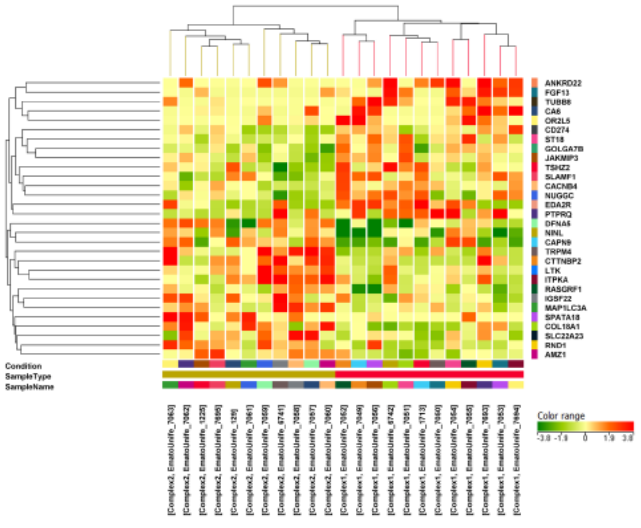

Figure 1. Heat-map representation of the average expression of the 30 most differentially expressed gene between patients with and without unbalanced rearrangements. The colours of the genes represented on the heat map correspond to the expression values normalized on gene mean expression across all samples: green indicates down-regulated; red indicates up-regulated. Legend: Complex1 = without unbalanced rearrangements; Complex2 = with unbalanced rearrangements.

CO023

THE SUBTYPES OF COMPLEX KARYOTYPES COMBINED WITH THE IGHV MUTATIONAL STATUS PROVIDE PROGNOSTIC AND PREDICTIVE INFORMATION IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction: Complex karyotype (CK), defined by the presence of at least 3 chromosomal lesions, is a heterogeneous cytogenetic category associated with adverse prognosis in several hematologic malignancies also with target agents. Recently, Rigolin *et al.* has provided evidence that in chronic lymphocytic leukemia (CLL) CK with major structural abnormalities (CK2) at diagnosis negatively impact on the time to first treatment (TTFT) and overall survival (OS) (Rigolin GM, BJH 2018). However, it is unknown whether the prognostic strength of CK could be implemented when combined with stable markers such as the IGHV mutational status. In the present study, we assessed the prognostic and predictive role of the combination of CK subtypes and IGHV status in a large CLL series.

Methods: Stimulated cytogenetics with CpG+IL2 was performed in 550 CLL patients. According to Rigolin *et al.*, CK2 cases included unbalanced translocations, addition, insertion, derivative and marker chromosomes. All other CK were classified as type 1 (CK1). An IGHV gene sequence homology >98% was considered as unmutated (U-IGHV), as opposed to mutated (M-IGHV). Treatment was initiated according to the iwCLL guidelines. TTFT and OS were calculated from diagnosis to first treatment or death, respectively, or last known follow-up. Survival curves were compared with the log-rank test and p<.05 was considered as significant.

Results: Among the 550 patients, 203 were males, the median age at diagnosis was 66 years, 204 were U-IGHV, 62 harbored TP53 abnormalities, 110 a CK (22 CK1 and 81 CK2), 243 received at least one line of therapy and 95 died over a median follow-up of 5.7 years. Focusing on the 374 patients with cytogenetic and IGHV status assessed within 6 months from diagnosis, 61 (16%) harbored CK2, 153 (41%) CK1 or U-IGHV and 163 (43%) M-IGHV without CK2. We observed that subjects with CK2 had a shorter 5-year TTFT (35%, 43% and 86%, p<.0001) and OS (65%, 83%, 91%, p<.0001) compared to cases with CK1/U-IGHV, or M-IGHV without CK. These data were confirmed in multivariate analysis. The combination of these two markers also provides predictive information after first-line therapy (p<.0001). In particular, among 78 patients treated with FCR or BR none of the M-IGHV cases relapsed or died after a median follow-up of 27 months compared with the two other two subgroups (2-year PFS: 100%, 76%, 45%, p<.0001; 2-year OS: 100%, 94%, 81%, p<.0001). We also observed that patients can be stratified into 3 statistically different subgroups even when CK, in particular CK2, is dynamically acquired (p<.0001) during the course of the diseases due to genomic instability.

Conclusions: In this study, we demonstrated that the combination of CK subtypes and IGHV status provides important prognostic and predictive data in CLL. Patients with M-IGHV without any subtypes of CK showed an excellent outcome with chemotherapy, but new alternative therapies should be explored for patients with U-IGHV and/or CK2.

CO024

CLONOTYPE SHARING AMONG PATIENTS AFFECTED BY γδ T-CELL LYMPHOPROLIFERATIVE DISORDERSS

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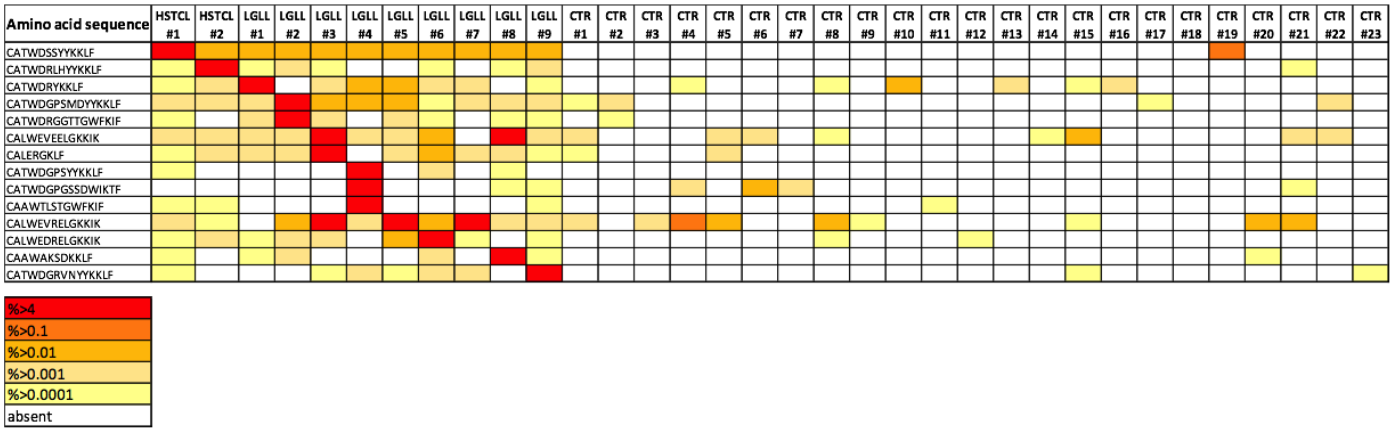
Introduction: Tγδ lymphocytes include a low percentage of circulating T cells (1-6%), usually displaying cytotoxic activity. These cells are considered "unconventional" for their expression of a T cell receptor (TCR) composed of a γ- and δ-glycoprotein chains. Tγδ-neoplasms include T-cell large granular lymphocytic leukemia (T-LGLL) and Hepatosplenic T cell lymphoma (HSTCL). The distinction of these diseases is sometimes very challenging. Although both conditions are characterized by the chronic proliferation of clonal Tγδ Large Granular Lymphocytes (LGLs), they have different prognosis and therapeutic approaches. Their etiology is not completely clarified yet, but it is supposed that an antigen can trigger the initial clonal cell expansion that is further maintained due to the lack of antigen clearance. In order to find evidence for common antigenic stimuli in Tγδ neoplasms, the aim of this work was to study TCR antigen-specific portion, evaluating the variable γ-chain complementary determining region 3 (CDR3) that represents the molecular signature (clonotype) of T-cell clone.

Methods: The study included 9 patients with TCRγδ-LGLL, 2 with HSTCL and a group of 23 healthy controls. Sanger sequencing was used for mutational analysis of STAT3 and STAT5b genes. Clonality was assessed by TCRγ gene rearrangement assay. CDR3 repertoire and frequency distribution of TCRγ gene rearrangements were obtained by Next-Generation Sequencing (NGS).

Results: By flow analysis, an increase of Tγδ cell percentage and, by TCRγ-gene rearrangement assay, the clonality was confirmed in all patients. Interestingly, by NGS, we demonstrated the presence of mono/biclonality in 7 patients and an oligoclonal pattern in the other 4, even if these latters appeared monoclonal by TCR rearrangement analysis. In these oligoclonal patients, STAT3/5b genes were more frequently wild type (3/4), while patients with mono/biclonal expansion (n=7) were mainly characterized by STAT3/5b mutations. CDR3 sequences of the immunodominant clones of γδ patients were analyzed for the presence of 100% homologous sequences in the other γδ cases and in healthy controls' group. We demonstrated that identical clonotypes were present at low frequency (>0.0001% of total reads/sample; Table 1) in almost all the other γδ patients, while they were usually undetected in healthy controls. Moreover, 2 different CDR3 sequences were found shared, each one by 2 and 3 different patients, respectively. Interestingly, the sequence of the immunodominant clone of one HSTCL was shared at a frequency >0.01% by all the other patients and only by 1 out of the 23 healthy controls.

Conclusions: These data confirmed the similarity between HSTCL and TCRγδ-LGLL, but also demonstrated a restricted clonotype repertoire among these patients with respect to healthy individuals, suggesting a non random clonal selection in T-LGLL and HSTCL disorders possibly driven by a common antigen.

Table 1. Homology assessment of immunodominant CDR3 sequences of γδ patients. NGS results about the frequencies of the main represented clonotypes of each patients (CDR3 sequences in the first column of the table) in the TCR repertoire of the other patients and controls. Coloured squares indicate that a sequence is present in the TCR repertoire of another sample. Different colours represent the different percentage by which the sequence is found in the sample. White squares indicate the absence of the sequence in sample TCR repertoire.



Benign Hematology (Red Cell Disease Thrombosis and Hemostasis)

CO025

RESULTS OF THE RANDOMIZED, DOUBLE-BLIND, PLACEBO-CONTROLLED, PHASE 3 HERCULES STUDY OF CAPLACIZUMAB IN PATIENTS WITH ACQUIRED THROMBOTIC THROMBOCYTOPENIC PURPURA (ATTP)

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Introduction: Acquired thrombotic thrombocytopenic purpura (aTTP) is a life-threatening autoimmune thrombotic microangiopathy. Episodes of aTTP are currently treated with plasma exchange (PE) to eliminate autoantibodies and to replenish ADAMTS13, and immunosuppression to address autoantibody formation. We report the results of a randomised, double blind, placebo controlled study of caplacizumab, a bivalent Nanobody, targeting the A1 domain of vWF, in addition to standard of care.

Methods: Patients with an acute episode of aTTP who had received one PE treatment were randomized 1:1 to placebo or 10 mg caplacizumab (daily), in addition to daily PE and corticosteroids. The study drug was given during the PE period and 30 days thereafter. If at the end of this period there was evidence of ongoing disease, such as suppressed ADAMTS13 activity, investigators were encouraged to extend the blinded treatment for a maximum of 4 weeks together with optimization of immunosuppression. All patients entered a 28-day treatment-free follow up period after the last dose of study drug.

Results: 145 patients were randomized (73 placebo, 72 caplacizumab). Compared to placebo, caplacizumab-treated patients were >50% more likely to achieve a platelet count response (platelet count normalization rate 1.55, 95% CI 1.10 – 2.20, p<0.01). During the study drug treatment period, treatment with caplacizumab resulted in a 74% reduction in TTP-related death, recurrence of TTP, or a major thromboembolic event (p<0.0001). During the overall study period, patients administered caplacizumab had a 67% reduction in disease recurrence (p<0.001). In all 6 caplacizumab-treated patients relapsed in the follow up period, ADAMTS13 activity was <10% at study drug stop, suggesting to continue treatment until resolution of the underlying disease. No caplacizumab-treated patients were refractory to therapy, while 3 placebo patients were (p =0.057). Caplacizumab treatment was associated with faster normalization of the 3 organ damage markers. The most common caplacizumab-related TEAEs were epistaxis, gingival bleeding, and bruising.

Conclusions: Treatment with caplacizumab reduced the time to platelet count response and resulted in a clinically meaningful reduction in aTTP-related death, recurrence of aTTP, or a major thromboembolic event during study drug treatment, as well as recurrences during the overall study period. The safety profile was favorable, with mucocutaneous bleeding the most frequently reported AE. Caplacizumab represents a novel treatment option for patients with aTTP.

CO026

CRYOPRESERVED PLATELETS FOR HLA-ALLOIMMUNIZED PATIENTS WITH BETA-THALASSEMIA TREATED WITH MYELOABLATIVE THERAPY

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Introduction: Cryopreservation of platelets (PLTs) is an effective technique to overcome issues associated with the short shelf life of liquid-stored PLT concentrates. Patients (pts) with transfusion-dependent thalassemias often develop anti-HLA antibodies (Ab), associated to platelet-transfusion refractoriness resulting in increased morbidity and mortality during aplasia after myeloablative therapy.

Methods: We describe the use of autologous and allogeneic cryopreserved PLTs as the sole support during thrombocytopenia in two beta-thalassemia major pts receiving myeloablative therapy: a 23-year-old-man and a 5-year-old-child. Luminex assay documented, for the adult pt, 28 anti-HLAA Ab, 49 anti-HLAB, 15 anti-HLAC, 2 anti-HLADQB1 and 1 anti-HLADRB1, and for the child, 9 anti-HLAA Ab, 21 anti-HLAB resulting in almost certain refractoriness to randomly selected PLT-transfusions. A cryopreservation program of PLTs was set: autologous for the adult pt and allogeneic for the child. Two autologous and and three allogeneic PLT-apheresis were collected using a separator with standard continuous-flow centrifuge technique and citrate anticoagulation. After collection, PLTs were irradiated and processed within 24 hours. The PLTs were split into freezing bags with a target concentration 1×10^{11} platelets/bag and cryopreserved with 5% DMSO. The bags were frozen by computer-controlled rate freezing and kept in liquid nitrogen at -196°C. Immediately before transfusion, PLTs were thawed in a warming water bath (37°C), diluted with citrate 10%, centrifuged and supernatant DMSO plasma removed. PLTs were resuspended in NaCl 0.9% solution. Their concentration was measured by an automated cell counter and microbiological analysis was performed at the end of manipulation. PLT surface and intracellular antigen expression was assessed, by flow cytometry, before cryopreservation and after thawing. Aliquotes were fixed and labelled with monoclonal Ab against CD61, CD42b and CD62P while vonWillebrand factor alpha granules were analyzed after permeabilization.

Table 1. Flow cytometry analysis of platelets.

	Before cryopreservation	After thawing
CD42 expression		
%	98.1	97.6
MFI	544.5	501.9
CD62P expression		
%	37.5	42.0
MFI	349.0	339.5
vWF content		
%	85.1	86.0
MFI	303.5	306.0

Results: The first pt transfused 300.5×10^9 PLTs on day +8 and 300×10^9 PLTs on day +12. PLT count rose from 9×10^9/L to 69×10^9/L and from 18×10^9/L to 97×10^9/L, respectively. The second pt transfused 202×10^9 PLTs on day +10 and 137×10^9 PLTs on day +12. PLT count rose from 18×10^9/L to 48×10^9/L and from 15×10^9/L 48×10^9/L respectively. No adverse reactions occurred. The pts had uneventful clinical courses. No minor or major bleeding occurred. The recovery of PLT count after thawing was about 95%. The cytometric analysis showed no significant differences before cryopreservation and after thawing.

Conclusions: We report safety and effectiveness of cryopreserved

PLT-transfusions in two thalassemia pts with numerous anti-HLA antibodies undergoing myeloablative therapy. This procedure can be implemented in the future, in selected cases, taking into consideration the economic impact and the work commitment.

CO027

THALIDOMIDE FOR THE TREATMENT OF SEVERE RECURRENT EPISTAXIS IN HEREDITARY HEMORRHAGIC TELANGIECTASIA: *IN VITRO* STUDY OF A NEW DRUG FORMULATION FOR A TOPICAL EFFECT

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Introduction. Hereditary hemorrhagic telangiectasia (HHT) is a genetic disease that leads to multiregional angiodysplasia. Severe recurrent epistaxis is the most common presentation, frequently leading to severe anemia. Therapeutic approaches are largely palliative. Since angiogenesis is involved in the pathogenesis of HHT, anti-angiogenic agents are supposed to be effective. In a previous open label, phase II, non-randomized, single-center study we observed that low-dose thalidomide (thal) was safe and effective in reducing epistaxis in HHT patients who did not benefit from other modalities of treatment, providing a rapid and enough durable clinical improvement without noticeable adverse events (Invernizzi *et al.*, Lancet Haematol 2015). However, the effect of thal is not permanent and a maintenance therapy may become necessary. Our aim was to develop a nasal formulation of thal for a local treatment as maintenance therapy in substitution of systemic administration.

Methods. Since thal is very slightly soluble in water, a powder formulation with the solubility enhancer cyclodextrin was produced. Thal powders were characterized for physico-chemical properties (particle size distribution, flow properties) and tested for *in vitro* dissolution rate, *in vitro* transport across rabbit nasal mucosa, insufflation and deposition in a human nasal model cast using different nasal powder devices.

Results: The particle shape by SEM analysis appeared as agglomerates with sharp edges. The thal nasal powders were stable products with suitable technological properties for nasal insufflation. Improved dissolution rate was found compared to that of the raw material. The transport through the rabbit nasal mucosa was insignificant; however an accumulation of the drug inside the membrane was found. This *in vitro* observation suggests a low likelihood of significant systemic absorption. The topical action on bleeding could benefit from the poor absorption and from the fact that about 2-3% of the thal applied on the nasal mucosa was accumulated within the tissue. Furthermore, residence time in the nasal cavity was prolonged by the mucoadhesive action provided by the nasal powder. Powders were completely emitted from all devices tested. A study generating the powder plume with insufflation devices into a silicone human nasal cast showed homogeneous deposition of the powder in the vestibular, pre-turbinate and turbinate regions.

Conclusions. These results support the suitability of thal/cyclodextrin powders for nasal application. We expect that the systemic availability of thal can be lower after nasal application, thus potentially avoiding the systemic untoward effects. We also hypothesize that the local application by nasal insufflation could improve its efficacy. After a tolerability preclinical study in animal, we plan to perform clinical investigations in order to assess the safety and pharmacokinetics of the new formulation.

Supported by Telethon Grant GGP 13036

CO028

HFE SNVS (SINGLE NUCLEOTIDE VARIANTS) IN IDIOPATHIC ERYTHROCYTOSIS: A REAL-LIFE EXPERIENCE OF A SINGLE CENTER

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Introduction: Erythrocytosis is characterized by persistently raised haemoglobin (HB) and hematocrit (Ht) levels. Differential diagnosis includes Policitemia Vera (PV), secondary - due to different ethiologies- and idiopathic erythrocytosis (IE). Recently, Randi *et al.* demonstrated the prevalence of HFE single nucleotide variants (SNVs) in patients with IE postulating a possible link between Haemochromatosis genes and erythrocytosis. The most frequent HFE SNVs are C282Y (rs1800562 G>A) and H63D (rs1799945 C>G) reported at allele frequencies of about 13% and 4% in caucasian countries.

Patients and Methods: We studied 16 patients with IE for the H63D, C282Y and S65C SNV in Haemochromatosis genes, using allele-specific real time-PCR. We previously ruled out the possibility of a diagnosis of PV (performing JAK2 mutation analysis for both V617F and exon 12 variant and bone marrow biopsy) and we investigated possible causes of secondary erythrocytosis with an appropriate algorithm (EPO level, chest and abdomen imaging, spirometry, venous p50 of HB, arterial blood gas analysis). EPOR, VHL, PHD2 and HIF2A mutations were studied to screen for hereditary erythrocytosis. None of the patients fulfilled the criteria of Hereditary Haemochromatosis (HH). Patient characteristics are summarized in Table 1.

Results: Of 16 patients with IE, 5 were carriers of the H63D, 1 of C282Y and 1 of S65C SNV of the HFE gene (44% of our cohort). Interestingly, two H63D heterozygous patients were father and son. They were all males of a median age of 54 years (range 18-64). The prevalence of HFE SNVs in our cohort of patients with IE is higher than expected for H63D and S65C in caucasians (32% vs 13% for H63D and 6.25 vs 1.5 % for S65C), while the allele frequency of C282Y SNV was similar to controls (6.25 vs 6%). Of note, the patient with the S65C SNV also had a PHD2 polymorphism PHD2p.C127S (rs1209790), whose role in erythrocytosis is not clear.

Conclusions: The relationship between IE and HFE SNVs is unclear. The increased prevalence of HFE SNVs in patients with IE may indicate an effect of impaired iron metabolism on erythropoiesis. Further studies –including the molecules involved in iron storage pathway- in a larger cohort of patients affected by IE are warranted to clarify the link between HFE genes and idiopathic erythrocytosis.

Table 1. Patients characteristics.

HB	ht	Age(y)	epo	HFE SNVs
18	53.5	18	7.41	H63D/wt
18.1	53.7	44	12.3	H63D/wt
18.2	52	52	6.1	wt
18	51	48	15.9	wt
18	52	62	13.5	wt
17.1	51	50	8.8	wt
18.8	54.7	61	5.4	H63D/wt
18	51.4	54	18.2	wt
18.8	57.3	63	7.1	H63D/wt
17.1	51.7	56	10.1	wt
19	55	56	12.5	wt
19	55	42	28,6	C282Y/wt
18.2	55.4	64	15.5	H63D/wt
17.3	53.3	42	10	wt
18.1	55.7	58	13.2	wt
18.9	55	43	7.9	S65C/wt

HB: hemoglobin; **ht**:haematocrit; **y**:years; **epo**: erythropoietin;
HFE SNVs : HFE gene Single nucleotide variants

CO029

ROLE OF OXYGEN SENSING PHD2 GENE VARIANTS IN ERYTHROCYTOSIS: REPORT OF THREE CASES

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Introduction: Hereditary erythrocytosis (HE) has been associated with PHD2 mutations (prolyl hydroxylase domain-containing protein 2 also called EGLN1), member of the complex pathway of "oxygen sensing" genes. Mutations in PHD2 gene, leading to its decreased hydroxylation ability, upregulation in the activity of HIF-1α and subsequent increase of red cell mass, have been previously reported. The PHD2p.C127S variant is frequently observed among Tibetans with D4E in cis (overall prevalence of this haplotype is 88.6% at altitude above 3000m), in linkage disequilibrium with other missense mutations (in particular HIF-2α/EPAS1). Surprisingly, the combined PHD2/HIF-2α variants result in a gain-of-function effect that blunts the hypoxic response, providing a molecular mechanism for the observed protection of Tibetans from erythrocytosis at high altitude. In normoxic conditions and in low-landers, the PHD2p.C127S variant could lead to increased erythropoiesis as reported by *in vitro* studies and the literature. Our aim was to investigate PHD2 gene in a cohort of patients affected by erythrocytosis.

Patients and Methods: Eighteen patients with erythrocytosis (17 males and 1 female with a median age of 54 years, range 18-76, Table 1) were studied for HE genes mutations by Sanger sequencing (EPOR exon 8, VHL coding region, PHD2 exon 1-3 and HIF2A exon 12). We previously ruled out the possibility of a diagnosis of PV (negative JAK2 mutation analysis for both V617F and exon 12 variant, and bone marrow biopsy) and we investigated possible causes of secondary erythrocytosis with an appropriate algorithm (EPO levels, chest and abdomen imaging, spirometry, venous p50 of HB, arterial blood gas analysis).

Results: Sequencing of HE genes identified 3 carriers of PHD2 variants in 18 patients (17%) with erythrocytosis. One patient had a missense heterozygous mutation (PHD2p.I269N), not previously described, together with a heterozygous mutation in the haemochromatosis gene H63D, previously reported as possible cause of erythrocytosis. Of note, his father died with a diagnosis of PV in the pre-JAK2 era and one of his two daughters also presents "erythrocytosis". Sequencing of PHD2 is ongoing in 4 first-degree relatives to establish the role of this mutation in erythrocytosis etiology. Interestingly, two further patients carry the missense heterozygous variant (PHD2p.C127S) previously reported in Tibetan population, whose role in patients with erythrocytosis is not totally clear.

Conclusions: We report a new PHD2 mutation possibly implicated in HE. Its effects in combination of H63D heterozygosity status may further explain erythrocytosis in our patient. PHD2 sequencing in the patient's relatives may clarify the role of this new mutation. Moreover, further studies are warranted to establish the role of the oxygen sensing PHD2p.C127S variant in non-highlanders Europeans and its effect on erythropoiesis.

Table 1.

PHD2	HB	ht	Age (y)	epo
PHD2p.C127S	17.9	51.5	76	8
PHD2p.C127S	18.9	55	43	7.9
c.806 t>a,p. I269N	17.1	54	55	8

HB: hemoglobin; **ht**: haematocrit; **y**: years; **epo**: erythropoietin

CO030

COMBINED ORAL ADMINISTRATION OF ANALGESIA AND ANXIOLYSIS FOR PAIN ASSOCIATED WITH BONE MARROW ASPIRATION AND BIOPSY

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Bone marrow aspiration and biopsy (BMAB) is a painful procedure, and the commonly adopted local infiltration anesthesia (LIA) with lidocaine is unable to relieve the pain during the most uncomfortable phases, or the anticipatory anxiety related to pain recalling thereafter. As there are no formal guidelines for adding a sedoanalgesic premedication before beginning the BMAB, many combinations have been adopted by several authors. Our randomized and patient blinded trial aimed to evaluate, as primary end point, the efficacy and safety of opioid and benzodiazepine agent combination plus LIA in patients who underwent BMAB for hematological malignancies. Two secondary end points were: 1) to define if patients who already underwent to BMAB without LIA prefer sedoanalgesia; 2) to demonstrate if sedoanalgesia can influence the quality of the biological specimen harvested. Patients were randomly assigned into two arms for receiving either placebo plus LIA (standard group, 48,6%) or oral fentanyl citrate 200 mcg plus oral midazolam 5 mg in addition to LIA (combo-group, 51,4%) during BMAB. Pre-procedural anxiety and procedural pain were assessed according to the Numered Rating Scale (NRS: 0-10), dividing the time of the procedure into five intervals (T0, T1, T2a, T2b, and T3) and evaluating discomfort grade during each moment of procedure in both groups. Cognitive function was measured before and 30 minutes after the procedure. Possible side effects were recorded, as well as the adequacy of tissue samples harvested. A telephone interview was performed 24 hours later. A total number of one-hundred-sixteen (n =116) were enrolled in the study. Nine (n = 9) patients did not meet inclusion criteria and were excluded. Fifty-two (n = 52) patients were randomized and assigned to standard group and fifty-five (n = 55) to combo group (Figure 1). At T2b and T3 (corresponding to the biopsy time and time after the biopsy, respectively) there was a significantly lower ($p< 0.05$) perception of pain in the patients who received sedoanalgesia (combo-group) compared to those who did not (standard group). Moreover, 100 % of the patients in combo group who had previously undergone this procedure without premedication reported that they would prefer sedoanalgesia for the subsequent procedures, thus confirming the effectiveness of this combination also in relieving anticipatory anxiety. Finally, the histological specimen was found to be high in quality, as defined by standards. Administration of oral analgesia and anxiolysis is a safe and feasible option to be used in outpatient setting; sedoanalgesia is very effective in reducing pain during the biopsy and it diminishes the anticipatory anxiety related to a painful procedure. Patients should have the possibility to choose between local anesthesia alone or sedoanalgesia plus local anesthesia.

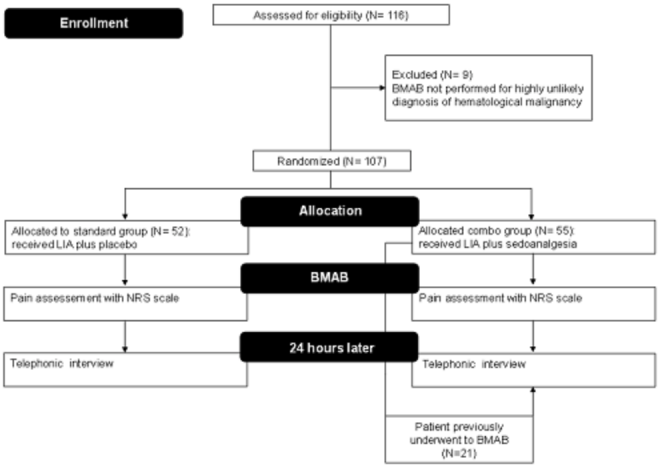

Figure 1.

CO031

EFFICACY AND SAFETY OF OPEN-LABEL CAPLACIZUMAB IN PATIENTS WITH EXACERBATIONS OF ACQUIRED THROMBOTIC THROMBOCYTOPENIC PURPURA DURING THE PHASE III HERCULES STUDY

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Introduction: The efficacy and safety of caplacizumab in patients with acquired Thrombotic Thrombocytopenic Purpura (aTTP) have been demonstrated in a single-blind phase 2 study (Peyvandi *et al.*, N Engl J Med 2016, 374(6):511-522) and confirmed in a double-blind phase 3 study (HERCULES; Scully *et al.*., Blood 2017 130:LBA-1).

Methods: In case of a recurrence during the double-blind (DB) treatment period, patients were switched to open-label (OL) caplacizumab, together with re-initiation of daily plasma exchange (PE) and immunosuppression, while maintaining the blind for the initial treatment allocation. Herein we present the efficacy and safety results of patients who received OL caplacizumab in the HERCULES study.

Results: Of the 145 randomized patients (73 to placebo and 72 to caplacizumab), 31 patients experienced an exacerbation during the DB period, 28 in the placebo group and 3 in the caplacizumab group. Twenty-eight of them were switched to OL treatment with caplacizumab (26 of the 28 placebo-treated patients and 2 of the 3 caplacizumab-treated patients). By day 6, 81% of patients receiving OL caplacizumab achieved a confirmed platelet count response (i.e., platelet count $\geq 150\times10^9$/L confirmed by stop of daily PE within 5 days). There were no deaths. One patient (3.6%) experienced a TTP exacerbation, and 1 patient (3.6%) experienced a major thromboembolic event (vena cava thrombosis) during the OL treatment period. After the end of OL treatment, 3 patients had a recurrence of TTP and all 3 had ADAMTS13 activity <10% when the treatment was stopped. The safety profile in the OL caplacizumab group was consistent with that observed in the DB caplacizumab group. In total, 25 patients (89.3%) were reported with at least one treatment-emergent adverse event.

Conclusions: Open-label therapy with caplacizumab was efficacious in HERCULES study patients who experienced an exacerbation of aTTP during the double-blind treatment period. The safety profile was consistent with that observed in the double-blind period.

CO032

DYSFIBRINOGENEMIA IN PATIENTS WITH PLASMACELLULAR DYSCRASIA

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Introduction: Disorders of fibrinogen (Fg) are usually the result of genetic mutations that hesitate in reduced levels of protein (hypofibrinogenemia) or in an abnormal molecule (dysfibrinogenemia). However, plasma factors or microenvironment can determine an acquired defect. Our goal is to identify the cause of dysfibrinogenemia in a 65 y/o man, diagnosed because of a discrepancy between immunological Fg and functional Fg. Hereditary deficit is excluded, due to normal coagulation values tested a year ago. Patient never showed thrombotic or hemorrhagic diseases.

Methods: The patient was screened for monoclonal gammopathy (plasma-serum and urine immunofixation, bone marrow study, FLC serum assay). A mixing test (1:1 ratio) was made adding PNP (pooled normal plasma) to patient's plasma (PP) at 4°C and 37°C. Purified Fg from PNP (FgN) was added to PP and to purified Fg from PP (FgP), in different mixing ratio. Functional Fg was assessed before and after mixing tests. A control group of 20 MM patients was screened for dysfibrinogenemia in order to detect similarities with the patient.

Results: the band of monoclonal light chain k was identified near to the band of fibrinogen on plasma immunofixation. No clonality was identified on serum immunofixation maybe because k chains were binded to fibrinogen molecules, absent in serum assay. Bone marrow plasma cells (PCs) were 4-5% (80% of these PCs were monoclonal). No functional Fg correction was observed after PNP addition (1:1 ratio) or after addition of FgN to PP: patient's plasma inhibits normal functional Fg. No difference in tests made at 4°C and 37°C, so we could rule out an interaction between k light chain and glucose part of Fg molecule. FgN was added to FgP: by increasing the FgP proportion in relation to FgN, functional Fg value declined until 5:1 ratio was reached. Functional Fg reached a plateau as if saturation of interfering capacity was achieved. No alterations in platelet aggregation tests were observed, so an interaction at the fibrinogen's binding site for platelets IIb/IIIa glycoprotein was excluded. As no hemorrhagic events were reported by the patient, an impaired binding of fXIII to the Fg binding site or a FpA (fibrinopeptide A) impaired release were unlikely. Steroid treatment resulted in an almost complete correction of functional patient's Fg value and FLC k values rose as if k light chains were released from Fg molecules. We screened 20 MM patients for dysfibrinogenemia: in 4 patients we found TT elongation but normal functional Fg values (control group). FgN was mixed to Fg purified from control group's patients: no decreasing in functional fibrinogen was noticed.

Conclusions: Considering the unusual plasma immunofixation profile we supposed that the inhibitory effect on Fg molecules was induced by monoclonal light chain k. A specific test to assay interaction between k light chain and FgN will be necessary to surely prove dysfibrinogenemia related to monoclonal FLCs.

Stem Cell Transplantation

CO033

TNF–α ENHANCES HUMAN REGULATORY T CELL FUNCTION VIA TNFR2: A NEW STRATEGY TO IMPROVE GVHD PREVENTION

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Introduction: CD4+CD25+FOXP3+ regulatory T cells (Tregs) can suppress proliferation and effector functions of T cells, B cells, NK cells, and antigen-presenting cells. In hematopoietic stem cell haploidentical transplantation, Tregs effectively prevented GvHD mediated by CD4+ and CD8+ conventional T cells (Tcons) (Martelli MF *et al.*. Blood 2014). Treg transfer did not impair Tcon-mediated graft versus leukemia effect as relapse incidence was 12% in patients with high-risk acute leukemias in remission at transplant. Paucity of Tregs in the periphery and the complexity of *in vitro* expansion limit further improvements of Treg clinical use, especially in patients transplanted with active disease. We recently showed that TNF-α induces mouse Treg activation as it reduces GvHD lethality in mouse models. Here, we investigated whether TNF-α priming of human Tregs could improve transplantation outcomes.

Methods: Peripheral blood CD4+ and CD4+CD25+ T cells were isolated by imunomagnetic bead separation. They were incubated for 60 hours with TNF-α (20 ng/ml) and low-dose IL-2 (10 UI/ml). The expression of Treg markers and homing receptors was evaluated by FACS analyses. TNF-α receptor 2 (TNFR2)+ and TNFR2- Tregs were separated by sorting. *In vitro* suppression of Tcon proliferation was evaluated by CFSE assay.

Results: *In vitro* stimulation with TNF-α and low-dose IL-2 selectively up-regulated CD25 expression on CD4+FoxP3+ Tregs (p<.05), but not on CD4+FoxP3- cells. TNF-α-primed Tregs maintained FoxP3 expression and expressed higher levels of TNFR2 (p<.03). Compared with Tcons, Treg are known to express higher levels of skin homing receptors (such as CCR4 and CLA-4), but lower levels of the intestinal homing receptor α4β7-integrin. TNF-α priming up-regulated the expression of α4β7-integrin, and could improve Treg migration to the gut and GvHD prevention. TNFR2 expression defines a Treg subpopulation with maximal suppressive capacity. Priming with either IL-2 alone or in combination with TNF-α further enhanced the function of TNFR2+ Tregs. Finally, we developed an easily translatable *in vitro* system for improving Treg function by adding IL-2/TNF-α stimulation in their selection process. CD4+ T cells were primed with TNF-α and low-dose IL-2 before CD25 positive selection. IL-2/TNF-α primed Tregs expressed higher CD25 and suppressed Tcon proliferation more efficiently (Figure 1, p<.01). This quick, easy-to-use, selection system could improve Treg effects in clinical trials.

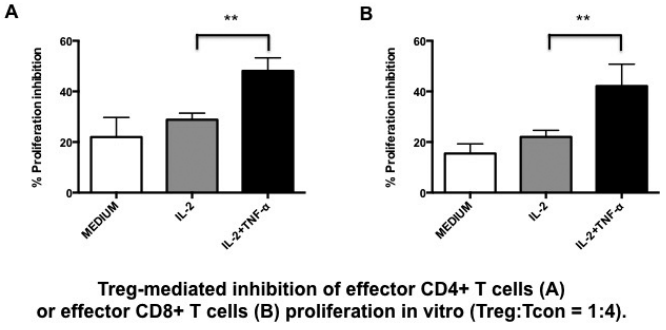

Figure 1.

Conclusions: We plan to confirm the present results in humanized preclinical models. This study could provide new insights on the role of TNF-α/TNFR2 pathway in human Treg function. Moreover, the present study may be critical to optimize the ongoing clinical trial, particularly in patients with active disease at transplant as it could allow for the infusion of higher doses of Tcons to enhance immune-reconstitution and leukemia killing without increasing the risk of GvHD.

CO034

ZINC SUPPLEMENTATION PROMOTES THYMIC FUNCTION AFTER DAMAGE BY STIMULATING ENDOGENOUS REGENERATION PATHWAYS

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The thymus, which is the primary site of T-cell development, is exquisitely sensitive to damage but also has a remarkable capacity for regeneration. Thymic production of new T-lymphocytes is crucial following hematopoietic stem cell transplantation (HSCT), therefore endogenous thymic regeneration is particularly important for successful clinical outcomes (Uzunel 2014, Stem Cells Dev). Zinc is the second most abundant metal in humans, and it is involved in cellular processes such as proliferation, inflammation, the response to oxidative stress, protection against pathogens, and wound healing (Prasad 2008 Mol. Med.). In a pilot clinical trial, we demonstrated that patients receiving oral zinc supplementation after autologous HSCT showed increased thymic function in the absence of adverse clinical events (Iovino 2018, Leuk Res). In order to elucidate the underlying mechanisms of this process, we used a murine model to evaluate the effect of zinc supplementation in thymic reconstitution after acute damage. Using a model of thymic damage caused by sub-lethal total body irradiation (SL-TBI, 550 cGy) on 6-weeks old WT C57BL/6J, we found that mice that received zinc in their drinking water (300 mg/Kg/day) showed increased thymic cellularity when compared to untreated age-matched mice, at days 3, 7, and 28 after SL-TBI (Figure 1a. Zinc: blue bars. Control: grey bars). We found an increased number of many thymic populations, including endothelial cells (EC, Figure 1b), which we have recently shown to be critical for endogenous thymic regeneration via their production of BMP4 (Wertheimer 2018, Sci Immunol). Notably, when stimulated *in vitro* for 24 hours with supraphysiological doses of zinc sulfate, ex vivo propagated ECs (exECs) were induced to produce BMP4 (Figure 1c), suggesting a likely mechanism by which zinc supplementation promotes thymic reconstitution. In conclusion, our data not only demonstrates that zinc supplementation can be an effective and safe clinical strategy to enhance T cell immunity, but also highlights an underlying mechanism by which this therapeutic approach operates.

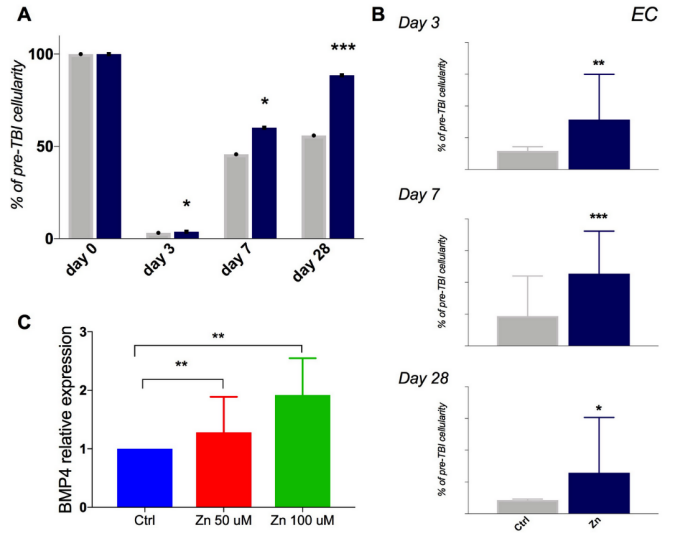

Figure 1.

CO035

SAFETY AND EFFICACY OF ANTI-LEUKEMIA CTL INFUSIONS FOR PREVENTION/TREATMENT OF LEUKEMIA RELAPSE IN HIGH RISK PATIENTS GIVEN HAPLOIDENTICAL HSCT

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Haploidentical hematopoietic stem cell transplantation (haplo-HSCT) represents an ideal platform for post-transplant cell therapy. In the past few years, our group has developed a procedure for the generation of donor-derived CTL, directed towards patient leukemic blasts, in accordance with good manufacturing requirements. We have documented the feasibility of inducing durable leukemia remission by transfer of donor-derived anti-leukemia CTL in two children affected by AML and ALL experiencing early relapse after haplo-HSCT. Considering that the follow-up of these children has reached 10 and 4 years respectively, it can be inferred that the CTL efficacy was not limited to the induction of a state of transient remission, but that they were able to eliminate LB, without any further infusion after the first year of treatment. In spite of the high number of anti-leukemia CTL transferred (range: 4-5.5×10^9 cells), no grade 2-4 toxicities, including emergence of severe graft-versus-host disease were recorded during follow-up. Since May 2016, after authorization by AIFA, we have enrolled 6 further patients (4 of whom have been treated) in protocols for the prevention or treatment of leukemia recurrence after haplo-HSCT with escalating CTL doses (from 104/kg to 8×10^6/kg) or (10-40×10^6/ kg), respectively. Pt#1ALL who was still MRD positive at transplantation received 12 infusions of escalating doses of CTL starting within 2 months after haplo-HSCT; she is in complete remission at 22 months post-HSCTand at 9 months from the last infusion. The remaining three patients started the CTL infusions later (4-7 months after haplo-HSCT), relapsed during the prevention phase and were enrolled in the recurrence protocol. Pt#2ALL relapsed after the first two infusions (bone marrow recurrence and hepatic and renal lesions). After combined treatment with blinatumomab and infusions of high dose CTL achieved morphological remission, complete chimerism, disappearance of hepato-renal lesions. The girl died of extramedullary disease progression and multi-organ failure. Pt#3ALL experienced extramedullary and medullary relapse at 13 months from haplo-HSCT, after 10 infusions of escalating doses of CTL; he received 7 infusions of high dose CTL combined with DLI. At present, he is in morphological remission, with non-quantifiable molecular MRD. Pt#4AML experienced bone marrow recurrence at 8 months post haplo-HSCT after two CTL infusions; he received 9 infusions of high dose CTL combined with Azacitidine and DLI; he is in cytofluorimetric remission, 20 months from haplo-TCSE. These preliminary results confirm that the control of leukemia recurrence with anti-leukemia CTL infusions is a complex scenario that involves multiple mechanisms and suggests that combined therapies with monoclonal antibodies, immunomodulatory drugs or DLI could enhance the GVL effect and induce remission.

CO036

MEMBRANE PROTEINS AND MICRORNAS IN EXTRACELLULAR VESICLES AS BIOMARKERS OF ACUTE GRAFT-VS-HOST DISEASE

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Introduction: Reliable biomarkers could be crucial to clinically define patients at high-risk of acute Graft-vs.-Host Disease (aGVHD). Extracellular Vesicles (EVs) may potentially become attractive biomarkers given their involvement in inflammation. In a preliminary study, we previously correlated 3 membrane proteins (CD146, CD31, CD140a) on EVs with the onset of aGVHD (Lia G. Leukemia 2017). To confirm our preliminary findings, we designed a prospective study to characterize EVs by their membrane protein expression profile and their content in MicroRNAs.

Methods: EVs are extracted from serum samples at given time-points (pre-transplant, on day 0, 3, 7, 14, 21, 28, 35, 45 and then monthly up to 1 year) by a protamine-based precipitation method and analyzed for the expression of membrane proteins (CD44, CD138, CD146, KRT18, CD120a, CD8, CD30, CD106, CD25, CD31, CD144, CD86, and CD140a) by flow-cytometry (Guava EasyCyte Flow Cytometer). Total EVs concentration, fluorescence distribution and percentage of positive EVs are evaluated for all membrane proteins as previously described (Lia *et al.* Leukemia 2017). MicroRNAs (miR100, miR92b, miR155, miR194) are extracted from EVs at specific time points (before transplant, on day 0, 7, 14, 28) by a commercial kit (miRNeasy mini kit Qiagen) and quantified by real time PCR after cDNA Reverse Transcription (miScript II RT kit Qiagen) as relative expression level compared to healthy donors. Odds ratio (OR) are calculated for each marker by logistic regression analysis.

Table 1. Association between EVs and acute GVHD. (A) Fluorescence membrane proteins and percentage of positive EVs as proportional change from baseline; B) miRNAs absolute quantification and C) miRNAs expression as proportional change from baseline.

A) EVs membrane protein			
		OR (95%CI)	p
CD146	Fluorescence	4,3283	,0081
	Percentage	3,5016	,0196
CD31	Fluorescence	,3051	,0008
	Percentage	,7742	,5598
CD44	Fluorescence	2,1872	,0390
	Percentage	2,3482	,0723
B) EVs miRNAs		**Absolute Value**	
		OR (95%CI)	p
miR100		1,272	0,437
miR155		2,108	0,064
miR92b		1,392	0,264
miR194		2,325	0,048
C) EVs miRNAs		**Proportional change from baseline**	
		OR (95%CI)	p
miR100		2,667	0,048
miR155		0,949	0,847
miR92b		1,615	0,388
miR194		1,934	0,248

Results: Thirty-five transplant patients have so far been enrolled and 17/35 patients (48.6%) developed grade II-IV aGVHD. Preliminary results on 21 patients have shown a correlation between CD146 (melanoma cell adhesion molecule, MCAM-1) and CD44 (homing-associated cell adhesion molecule, H-CAM) levels and increased risk of aGVHD (OR 4.3, p=0.008; OR 2.2, p=0.039), whereas CD31 (platelet endothelial cell adhesion molecule, PECAM-1) level was associated with a decreased risk of aGVHD (OR 0.31, p=0.0008). (Table 1A). Of note, biomarkers associated with aGVHD showed a consensual change in signal level (increased CD146 and CD44 levels, decreased CD31 level) from baseline up to aGVHD onset. MicroRNAs expression levels showed an association between upregulation of miR100 and miR194 and increased risk of aGVHD (Table 1B-C).

Conclusions: An association of 3 membrane proteins expressed on EVs and onset of aGVHD was observed. Of note, CD146, CD44 and CD31 belong to the Cell Adhesion Molecule Family and are crucial for endothelium and immune cells interactions. MicroRNAs play critical roles in several biological processes affecting T-cells. Hence, miRNAs are been investigated as potential biomarkers for GvHD. The functional role of miR-194 in GVHD pathogenesis remains to be determined (Gimondi S Exp Hematol, 2016) while miR-100 has been reported to limit neovascularization in the intestine during GvHD (Leonhardt F Blood 2013).

CO037

THYMIC FUNCTION AND T CELL HOMEOSTASIS AFTER HAPLOIDENTICAL ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Introduction: Post-transplant cyclophosphamide (PTCY) has expanded the application of haploidentical stem cell transplantation (haplo-HSCT). Thymic function may play a pivotal role in long-term clinical outcomes.

Methods: ETwenty-nine patients (median age 53, r 28-70) with hematological malignancies underwent haplo-HSCT with PTCY. Blood samples were collected before conditioning and at 1, 3, 6, 12, 18, 24 months after transplant. Analyses of CD4+ and CD8+ T-cell subsets by flow-cytometry were correlated with Real-Time PCR quantification of signal joint T-cell receptor excision DNA circles (sjTRECs), specific marker of naïve T-cells thymopoiesis. A) Naïve; b) central; c) memory; and d) revertant T cells were defined as follows: a) CD4+CD45RA+ CD62L+; b) CD4+CD45RO+CD62L+; c) CD4+CD45RO+CD27-; and d) CD4+CD45RA+/45RO+, respectively. SjTRECs real-time PCR was performed on genomic DNA extracted from sorted CD4 and CD8 T-cells. Associations between sjTRECs and T-cell subsets and trends over time were evaluated by Generalized Linear Models.

Results: Following PTCY induced T-cell depletion, a constant gradual increase in absolute numbers of all T-cell subsets and of sjTRECs from the first month up to two years post-transplant was observed. Table 1 summarizes: a) patient median counts of CD4+ and CD8+ T cell subsets at 1, 3, 6, 12, 15, 24 months; b) median values of sjTREC copies/100 ng DNA from sorted CD4+ and CD8+ T cells; c) median values of healthy donor T cell counterparts and sjTREC copies respectively. Overall, at two years, CD4 and CD8 T-cell levels and sjTRECs levels were lower than those observed in healthy donors. Molecular analysis of the sjTRECs kinetics was associated with the increase in CD4+ naïve T-cells (global p<0,008). This correlation clearly suggests that most of naïve T-cells derives from thymic re-education of donor precursor stem cells. Furthermore, an increase in revertant memory T-cells was also significantly correlated with sjTRECs kinetic (p 0,041 and <0.001 respectively). By contrast, central and effector memory T-cells showed a faster thymic-independent expansion. Importantly, sjTRECs levels and thymic dependent immune-reconstitution were higher in a cohort of 63 adult patients undergoing HSCT from HLA identical donors (data not shown). The impact of clinical parameters was evaluated on thymic function from 6 months after transplant onwards. By multivariate analysis, low baseline TRECs values, moderate-severe chronic GVHD, age older than 50 years old were significantly associated with low thymic output after haplo-HSCT.

Conclusions: Active thymic function despite age-dependent involution substantially contributes to T-cell reconstitution after haplo-HSCT. Lower production of sjTRECs as compared after HLA identical sibling transplants may partly be due to a higher degree of "mismatching" of MHC molecules during thymic re-education. Chronic GVHD and older age are significantly correlated with thymic activity.

Table 1.

	Time after HSCT (months)							
$CD4^+$ subset (count/μl)	**preTx**	**1**	**3**	**6**	**12**	**18**	**24**	**donors**
Naïve $CD45RA^+CD62L^+$	41,7	1,9	2,3	5,5	9,3	31,9	40,2	199,6
Revertant memory $CD45RA^+45RO^+$ $CD62L^+$	53,4	2,7	7,0	13,0	18,3	24,5	40,4	131,1
Central Memory $CD45RO^+CD62L^+$	82,9	17,6	50,7	89,8	127,0	202,8	197,6	161,9
Effector memory $CD45RO^+CD62L^-$	37,6	6,9	32,3	61,2	186,6	122,0	102,0	91,6
$CD8^+$ subset (count/μl)								
Naïve $CD45RA^+CD62L^+$	24,8	2,2	10,1	50,4	75,5	83,7	63,7	115,1
Revertant memory $CD45RA^+45RO^+$ $CD62L^+$	49,5	16,1	29,1	63,7	88,2	128,3	112,8	34,7
Central Memory $CD45RO^+CD62L^+$	25,4	29,5	13,5	36,0	70,3	96,7	52,5	17,8
Effector memory $CD45RO^+CD62L^-$	56,9	2,5	11,8	86,6	148,0	277,3	253,3	40,6
sjTRECs (copy/100ng gDNA)								
$CD4^+$	7,5	-	2,8	5,9	36,2	25,8	22,4	84,6
$CD8^+$	5,3	-	1,6	4,6	8,9	13,6	8,2	77,9

CO038

MULTIFACETED IMMUNE CHECKPOINT EXPRESSION AND SENESCENT MARKERS IMPAIRS BONE MARROW Vγ9Vδ2 T- CELL FUNCTION IN MULTIPLE MYELOMA PATIENTS

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Introduction: Malignant B cells are privileged targets of Vγ9Vδ2 T-cell recognition and killing *in vitro*, but this predisposition is lost *in vivo* due to multiple mechanisms mediated by tumor cells and the tumor microenvironment (TME). By interrogating the phosphoantigen (pAg) reactivity of bone marrow (BM) Vγ9Vδ2 T cells from multiple myeloma (MM) patients, we have unravelled a very early and long-lasting Vγ9Vδ2 T-cell immune dysfunction sustained by several mechanisms, including a multifaceted immune checkpoint (ICP) expression in the TME. Notably, single-agent PD-1 blockade is insufficient to recover anti-myeloma immune responses mediated by BM Vγ9Vδ2 T cells, indicating the existence of primary and adaptive resistance to ICP blockade.

Methods: The expression of ICP and senescent markers by BM Vγ9Vδ2 T cells in multiple myeloma patients was examined by multicolor flow cytometry. Vγ9Vδ2 T-cell functions was evaluated *in vitro* using the appropriate stimulation and combinations of different anti-ICP mAbs. Western blot analysis has been used to analyze the signal transduction pathways triggered by PD-1 (PI3K/Akt and Ras/MEK/ERK) and TIM-3 (JAK/STAT) in BM Vγ9Vδ2 T-cell after pAgs stimulation.

Results: We have observed a significant up-regulation of alternative inhibitory receptors (TIM-3 and TIGIT) in MM BM Vγ9Vδ2 T cells after pAg stimulation and PD-1 blockade. The combination of PD-1 and TIM-3 blockade improves the recovery of BM Vγ9Vδ2 T-cell proliferation. To investigate the molecular mechanisms implied in the alternative ICP up-regulation driven by single PD-1 blockade, we have analyzed the signal transduction pathways triggered by PD-1 (PI3K/Akt and Ras/MEK/ERK) and TIM-3 (JAK/STAT) in BM Vγ9Vδ2 T cells under resting conditions and after pAg stimulation. We have identified a remarkable down-modulation of pAKT, pJAK1 and pSTAT1, which may contribute to the exacerbation of Vγ9Vδ2 T-cell dysfuntion. We have also documented a significantly down-modulation of the transcription factor T-bet as an additional hallmark of BM Vγ9Vδ2 T-cell exhaustion. These senescent tumor-experienced Vγ9Vδ2 T cells with enhanced resistance to PD-1 blockade are phenotypically characterized by CD160 expression and lack of CD28 expression.

Conclusions: Deciphering the immune suppressive mechanisms involved in BM Vγ9Vδ2 T-cell anergy of MM patients can be very infor-

mative to develop effective interventions to fully exploit the immune potency of Vγ9Vδ2 T cells in B-cell malignancies.

CO039

GENOME WIDE METHYLATION IN HEMATOPOIETIC STEM CELLS AFTER ALLOGENEIC BONE MARROW TRANSPLANTATION

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Introduction: Allogeneic hematopoietic stem cell transplantation (AHSCT) is a curative therapeutic approach for different hematological diseases. Little is known, however, about the biology of HSCs after transplant in terms of homing, self renewal and differentiation. DNA methylation is involved in HSC stemness and commitment, thus potentially exerting a role in the reconstitution of hematopoietic system after AHSCT. We explored DNA methylation dynamics in bone marrow (BM) HSCs after transplant.

Methods: We enrolled 10 patients, respectively affected by AML (n=6), ALL (n=3), and HL (n=1), and receiving BM AHSCT. CD34+ cells were sequentially collected from BM of donors (t0) and matched recipients [day 30, 60, 120, 180, 360 (t1-t5)]; DNA was used for array-based methylation (Infinium HumanMethylationEPIC, Illumina). A schematic overview of the study design is reported in Figure 1.

Results: Genome wide methylation profiles of donors and recipients, interrogating 850.000 CpG sites, were generated. We firstly performed an unsupervised hierarchical clustering observing that, generally, each patient profiled into a specific methylation cluster; some donors clustered together, while others separated with their respective recipient. Of note, global methylation level was similar in all times (t0-t5), when considering all samples. Subsequently, to assess methylation changes after transplant, we compared methylation profiles of donor and recipient HSCs. First, we identified 12043 differentially methylated probes (DMPs) in t1 vs t0, mapping to 1857 genes (1380 hyper- and 477 hypo-methylated). Interestingly, hypo-methylated genes were involved in immune responses, resistance to host and allograft rejection, while hyper-methylated genes were enriched in cell remodeling, adhesion and cell activation. As expected, both hypo- and hyper-methylated genes enriched in function related to hematopoiesis, such as leukocyte activation and differentiation. Thus, 30 days after AHSCT, CD34+ cells consistently modified their methylation pattern. When we evaluated DMPs at other time points respect to donors, their number decreased indicating that methylation levels tend to normalize during transplant. To verify this, we evaluated: (i) differentially methylated genes both in t1 and t5 against t0, representing a long term modification, and (ii) differentially methylated genes at t1, but not in t5, against t0, having a short term effect only present in t1. We identified only 270 differentially methylated genes, while the majority (n=1699) showed a methylation profile similar to donors.

Conclusions: Our study shows that HSC methylation pattern consistently changes in an early time after AHSCT, possibly as a consequence of the adaptation of donor cells in recipient BM niche. Moreover, after a year, HSCs prevalently restore methylation patterns similar to donor cells at baseline. Ongoing studies on status and dynamic of HSC methylation could define its role in AHSCT outcome.

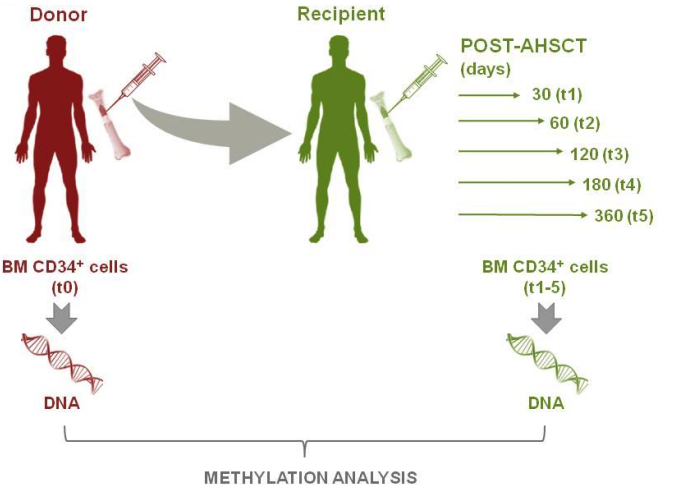

Figure 1.

CO040

ABCA1: AN UNEXPECTED ROLE IN Vγ9Vδ2 T-CELL ACTIVATION BY DENDRITIC CELLS

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A very peculiar feature of Vγ9Vδ2 T cells is their ability to recognize pyrophosphorylated isoprenoids [i.e, phosphoantigens (pAgs)], generated in the mevalonate pathway of mammalian cells. Isopentenyl pyrophosphate (IPP) is the prototypic pAg recognized by Vγ9Vδ2 T cells. Tumor cells and dendritic cells (DCs) are privileged targets of Vγ9Vδ2 T cells because they produce significant amounts of IPP which can be boosted with zoledronic acid (ZA), the most potent aminobisphosphonate clinically available and a strong inhibitor of farnesyl pyrophosphate synthase in the Mev pathway. ZA-treated DCs generate and release in their supernatants picomolar IPP concentrations which are sufficient to induce the activation of Vγ9Vδ2 T cells. We have recently shown that the ATP-binding cassette transporter A1 (ABCA1) plays a major role in the extracellular release of IPP from ZA-treated DCs. This novel ABCA1 function is fine-tuned by physical interactions with IPP, apolipoprotein A-I (ApoA-I) and butyrophilin-3A1 (BTN3A1). IPP binds to ABCA1, BTN3A1, and apoA-I, further promoting interactions between these molecules. We speculate that the aim of this mènage à trois is two-fold: the first is to extend the range of immune regulation to Vγ9Vδ2 T cells that are not in close proximity to pAg-presenting cells; the second to protect pAg-presenting cells from apoptosis due to intracellular accumulation of the pro-apoptotic ATP analog AppI. A BCA1 can work as a safety valve instructed to avoid that Vγ9Vδ2 T-cell activation is prematurely terminated by the apoptotic death of pAg-presenting cells. These data further strengthen the close relationship between lipid metabolism and immune function.

Molecular Hematology

CO041

ETNK1 MUTATIONS INCREASE MITOCHONDRIAL ACTIVITY AND PROMOTE DNA DAMAGE THROUGH ROS PRODUCTION

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Introduction: Atypical chronic myeloid leukemia (aCML) is a clonal disorder belonging to the MDS/MPN syndromes. About 13% of aCML cases carry somatic mutations in ETNK1 gene, encoding for H243Y, N244S and G245V substitutions. In ETNK1-positive aCML primary samples the intracellular level of phosphoethanolamine (p-ET), the product of the ETNK1 kinase, was 5.2-fold lower than in the non-mutated ones. Despite this evidence however, the functional role of ETNK1 mutations remained largely unexplained. Since p-ET pathway significantly contributes to the mitochondrial level of phospholipids, we focused our attention on mitochondrial activity.

Methods: The CRISPR/Cas9 system was used on 293 Flp-In™ cell line to generate clones carrying heterozygous N244S mutation and homozygous ETNK1 functional deletion (KO cells). Single-cell FACS sorting was adopted to isolate single-cell clones, and their genotype was checked by performing deep-sequencing analysis. MitoTracker™ Red CMXRos was used to visualize mitochondrial activity. CellROX™ Green Reagent was used to assess ROS production. ATP levels were quantified by using ATPlite Luminescence Assay System. DNA damage was detected by quantifying γ-H2AX foci formation in cell lines, or γ-H2AX protein expression in patient samples. Mutation frequency was determined by 6-thioguanine assay. Clonal evolution reconstruction was performed by methylcellulose colony assay.

Results: In both N244S and KO cells, mitochondrial morphology changed from an elongated, tubular shape to a round, swollen one. Moreover, N244S and KO cells showed a significant increase in mitochondrial activity (1.78 and 2.13 fold increase, respectively; p=0.0096 and p=0.0050) compared to WT, and also in ROS (1.66 and 1.74 fold increase, respectively; p<0.0001) and ATP production (1.67 and 1.68 fold, respectively; p<0.0001 and p=0.0082). γ-H2AX analysis revealed a higher number of foci (p<0.0001) in N244S and KO cells (2.60±0.22 and 2.89±0.27) compared to WT (0.56±0.08). A similar increase in γ-H2AX (p=0.0037) was present in primary aCML patients samples carrying ETNK1 mutation compared to ETNK1-WT ones. In line with these data, 6-thioguanine assay showed a higher mutation rate in N244S and KO cells ($8.09\times10^{-7}\pm9.6\times10^{-8}$ and $8.20\times10^{-7}\pm1.28\times10^{-7}$; p=0.0060 and p=0.0264) compared to WT ($2.98\times10^{-7}\pm8.2\times10^{-8}$). The hierarchical reconstruction of somatic mutations in ETNK1-mutated aCML patients revealed that ETNK1 variants invariably occur very early in the evolution history of aCML patients.

Conclusions: Our results show that impairment of ETNK1 function causes an increase in mitochondrial activity, which in turn leads to increased production of ROS driving the accumulation of DNA mutations. Since ETNK1 mutations are a very early event in the history of the disease, we hypothesize that ETNK1 could contribute to the onset of aCML through the activation of a mutant phenotype, which in turn would accelerate the accumulation of further oncogenic mutations.

CO042

MOLECULAR PROLIFE BY NEXT GENERATION SEQUENCING OF ACUTE MYELOID LEUKEMIA WITH NORMAL KARYOTYPE: CLINICAL RESULTS FROM THE PROSPECTIVE TRIAL 02/06 OF THE NORTHERN ITALY LEUKEMIA GROUP (NILG)

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Background. Treatment strategies for Acute Myeloid Leukemia (AML) patients are based on clinical features and cytogenetic/genetic profile of each patient. For patients with a normal karyotype (NK) AML the final risk classification is also defined by the identification of somatic mutations that have been recently described and impact on patient survival.

Aims. To describe the clinical impact of the gene mutation profile of NK AML patients treated within the prospective NILG trial 02/06 [ClinicalTrials.gov Id: NCT00495287].

Methods. Genetic profiling of the 572 patients enrolled into the 02/06 trial was prospectively obtained with standard approach and subsequently with Next Generation Sequencing (NGS) for NK-AML with available material (207 out of 250). Two commercial NGS kits were applied: Trusight Myeloid panel (Illumina) and Sophia Myeloid Solution (Sophia genetics) investigating 54 and 30 gene regions, respectively.

Results. The median age of the 207 studied NK AML was 52 years (19-74) and 30% of them had >50.000 White Blood Cell (WBC) count. The 5-year overall survival (OS) and Disease Free Survival (DFS) were 52% and 51%, respectively. The most frequently mutated genes in this cohort were NPM1 (50%) followed by DNMT3A (38%), FLT3-ITD (26%), CEBPA (20%), (single mutated, sm, 9%, double mutated, dm, 11%), TET2, RUNX1, IDH2, FLT-TKD, ASXL1, IDH1 (16-10%); other genes were mutated below 10%. We verified whether the incidence of co-occurring mutations was different among NPM1mutated (NPM1m) and NPM1 wild type (NPM1wt) groups of patients. DNMT3A, FLT3-ITD, FLT3-TKD, IDH1, PTPN11 and RAD21 mutations were associated to the NPM1m group (p<0.05) while CEBPAdm, RUNX1, IDH2, ASXL1, SRSF2, MLL-PTD, STAG2, BCOR, GATA2, WT1, SF3B1, U2AF1 mutations were associated to the NPM1wt group. Univariate analysis showed that FLT3-ITD negatively affected the OS of both NPM1m and NPM1wt patients while DNMT3A, RUNX1, TET2, IDH1, NRAS, U2AF1, determined a worse OS in the NPM1wt group (p<0.05). In this latter, CEBPAdm was associated to a favorable clinical outcome while ASXL1 had an adverse effect in the absence of RUNX1 mutation. The 5-year OS of NPM1m group with or without FLT3-ITD were 38% and 72%, respectively (P=0.0002, Figure 1A), while the 5-year OS of NPM1wt group with or without any of the above described prognostically detrimental gene mutations were 26% and 72%, respectively (P<.0001, Figure 1B). Allogeneic stem cell transplantation was associated to a better OS and DFS in both the NPM1m and NPM1wt cohort. Multivariate analysis confirmed the results except for IDH1 and NRAS that did not affect the NPM1wt patients outcome; the beneficial effect

of transplant was confirmed only in the NPM1m group.

Conclusion. Molecular profiling of NK AML is crucial for a precise definition of the relapse risk in AML patients, particularly those lacking the NPM1 mutation. High throughput NGS provides timely information on a growing set of genes and may improve treatment strategies

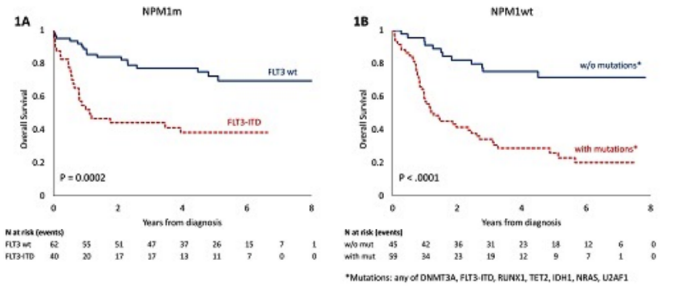

Figure 1.

CO043

MINIMAL RESIDUAL DISEASE (MRD) DETECTION BY DIGITAL-DROPLET-PCR (DDPCR) IN LYMPHOID MALIGNANCIES

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Background. Analysis of minimal residual disease (MRD) has an important clinical impact in different lymphoid malignancies. Real-time-quantitative-PCR (RQ-PCR) is the most widely used method. Digital-droplet PCR (ddPCR) has several theoretical advantages, allowing for the absolute quantification of target DNA molecules and avoiding the need of a reference standard curve. We evaluated the use of ddPCR for MRD monitoring and compared it head-to-head with RQ-PCR in 175 patients with different lymphoid malignancies.

Methods. Genomic DNA was screened by PCR amplification. MRD was evaluated in the bone marrow and/or peripheral blood by RQ-PCR, according to the guidelines (Van der Velden, 2007) and by ddPCR as described (Della Starza, 2016; Cavalli, 2017).

Results. Overall, we evaluated 504 follow-up (FU) samples: 231 were acute lymphoblastic leukemia samples, 133 were early stage follicular lymphoma samples, 24 were mantle cell lymphoma samples and 116 were chronic lymphocytic leukemia samples. Overall, MRD detection was concordantly positive or negative in 78% (393/504) of FU samples ($r = 0.78$, $p<0.0001$), while 22% (111/504) were identified as discordant (Table 1). Most of the discordances occurred in FU samples with a low level of disease - positive not quantifiable (PNQ) or negative - and did not appear to cluster in specific disease subsets. Among the 89 RQ-PCR-PNQ samples, in 31 (35%) ddPCR was able to provide a reliable quantifiable result (Q), whereas in 26 (29%) it proved negative. On the other hand, among the 64 ddPCR PNQ samples, 28 (44%) scored negative by RQ-PCR, whereas only 4 (6%) were Q ($p<0.0001$). Focusing on the negative samples, among the 246 RQ-PCR-negative, ddPCR was able to recover 47 positive samples, of which 19 were Q; among the 228 ddPCR-negative samples, RQ-PCR recovered 29 positive samples, of which only 3 were Q ($p=0.0082$) (Table 1). In summary, 22/504 samples (4%) were major discordances: 19 were RQ-PCR-/ddPCR Q and 3 were RQ-PCR Q/ddPCR-; 18% (89/504) were minor discordances: 31 RQ-PCR-PNQ/ddPCR Q, 26 RQ-PCR PNQ/ddPCR-, 4 ddPCR PNQ/RQ-PCR Q and 28 ddPCR PNQ/RQ-PCR-. Overall, the use of ddPCR significantly reduced the proportion of PNQ samples compared to RQ-PCR (64/504 [13%] vs 89/504 [18%], respectively) ($p=0.03$), increasing the proportion of Q samples (212/504 [42%] vs 169/504 [33.5%], $p=0.006$).

Conclusions. ddPCR and RQ-PCR exhibited a good correlation in all disease entities investigated. Most discordances were recorded in samples with low MRD levels, in which ddPCR was able to identify a quantifiable disease more reliably than RQ-PCR. ddPCR may be considered as an alternative tool for MRD assessment in lymphoid malignancies and it is currently being investigated in the context of the GIMEMA trials to define whether it may substitute RQ-PCR in routine clinical laboratories.

Table 1. Overall concordance and discordances between RQ-PCR and ddPCR. In grey, cells with discordant samples.

		RQ-PCR			
		Neg	PNQ	Q	Total
ddPCR	Neg	199	26	3	228
	PNQ	28	32	4	64
	Q	19	31	162	212
	Total	246	89	169	504

CO044

ERYTHROPOIESIS HAS AN UNEXPECTED REGULATOR: PROTEIN KINASE CK2

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Introduction: CK2 is a Ser-Thr protein kinase well known for its ability in sustaining cell survival and proliferation. It consists of two catalytic (α) and two regulatory (β) subunits, although these components can also work separately. Despite CK2 regulates key signaling pathways for hematopoiesis, its role in blood cell development is still undefined. To fill this gap, we have generated a conditional knockout (KO) mouse model for CK2β in the hematopoietic system. CK2β KO led to birth lethality, thus the analysis was performed during gestation. CK2 loss caused a depletion of all hematopoietic lineages, markedly of the erythroid population; moreover, CK2β KO led to a down-modulation of protein members of the Epo-receptor signaling, in particular of GATA-1 transcription factor. Here, we aimed at dissecting the molecular determinants of CK2β-driven erythroid differentiation. To confirm the hypothesis of a functional relationship between GATA-1 and CK2β we explored the erythroid differentiation *in vivo* in CK2β KO erythroid cells as well as in an *in vitro* model of erythroid differentiation based on G1E-ER mouse proerythroblast cells bearing a β-estradiol-inducible form of GATA-1.

Methods: G1E-ER cells were exposed to β-estradiol with or without CK2 inactivation with either the inhibitor CX-4945 or siRNA against CK2β; bortezomib was add to determine GATA-1 turnover. We used flow cytometry to study erythroid differentiation and cell cycle. mRNA expression levels were evaluated by RNA sequencing and by RT-PCR. WB was performed to assess protein amount; immunofluorescence to investigate GATA-1, CK2β and HSP70 cell localization.

Results: We found that CK2β KO or inactivation during erythroid differentiation caused a reduction of more mature TER119+ cells; also, CK2 blockade impacted the cell cycle in a maturation stage-dependent fashion. We observed a down-modulation of STAT5, AKT and GATA-1 protein levels and an impairment of GATA-1 transcriptional activity. Nuclear GATA-1 resulted lowered and its distribution changed. Treatment with Bortezomib restored GATA-1 levels. As CK2 influences HSP70, HSP90 and CDC37 ability to stabilize client proteins, we considered a possible indirect role of CK2 in GATA-1 protein turnover control. Indeed, we observed a decrease in these chaperone expression, upon

CK2 inactivation, which could account for GATA1 instability. However, GATA-1 and CK2β also colocalized in the nucleus in presence of the β-estradiol suggesting a possible direct mechanism in CK2-mediated control of this transcription factor.

Conclusions: CK2β is essential for erythroid maturation, regulating STAT5, AKT and GATA-1. Further research will clarify if CK2β subunit could influence these factors in an indirect or direct mechanism.

CO045

COMPORTAMENTO DELLE MUTAZIONI DRIVER E NON DRIVER NEI PAZIENTI CON MIELOFIBROSI IN TRATTAMENTO CON RUXOLITINIB

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Background: Introduction of ruxolitinib changed the outcome of patients with myelofibrosis (MF), offering longer survivals. Nevertheless, 50% of patients loss response; in some cases, this phenomenon has been ascribed to driver and non-driver mutations, but with conflicting results: Patel *et al.* (Blood 2015) reported that having >3 mutations well correlated with shorter time to discontinuation and overall survival, whereas in the COMFORT-II study the MF-associated mutations did not correlate with response, survival or discontinuation probability (Guglielmelli, Blood 2014).

Aims: in order to investigate if ruxolitinib could play any role in changing the mutational landscape in MF, we assessed the 3 driver and 8 non-driver mutations in 36 MF patients; all were assessed a diagnosis, 19 also after 12 months of ruxolitinib, and other 4 after hydroxyurea.

Methods: In addition to assessment of the driver mutations (JAK2, CALR, MPL), a PCR plate with pre-spotted primers able to detect 8 non-driver mutations was designed (Custom qBiomarker Somatic Mutation PCR Array® - Qiagen, Italy). ASXL1, EZH2, DNMT3A, IDH1, IDH2, SRSF2, TET2, TP53, for total 38 hot-spot sites, were assessed.

Results: JAK2 was mutated in 70% of cases, CALR in 20%, whereas 10% of cases were triple-negative. The median OS was significantly longer for primary MF (160 months) vs post-ET (80 months) or post-PV MF (35 months)(p=0.03), and for CALR- vs JAK2-mutated patients. At the last follow-up, 4 patients (11%) progressed to AML, and 12 (33%) died. The non-driver mutations were found at diagnosis in 33% of cases receiving ruxolitinib and in one/4 patients treated with hydroxyurea. Considering both driver and non- driver mutations, 24 cases (67%) were mutated, with 16 cases carrying one, and 10 two mutations. The most frequently detected mutations belonged to the methylation pathway (DNMT3A, IDH, TET2 = 75%), followed by TP53 (17%), SRSF2 (8%), ASXL1 (8%), and EZH2 (8%). During treatment, JAK2 allele burden remained stable, whereas non-driver mutations changed in 13 cases: 9 acquired a new mutation (DNMT3A in 5, IDH2 in one, and TP53 in another one) while other 4 lost mutation. None of the CALR-mutated cases carried non-driver mutations. In the 4 cases treated with hydroxyurea, one acquired the TP53 and another one the DNMT3A mutation. On the other hand, 4 cases in the group of ruxolitinib, and none in the group of hydroxyurea lost mutations present at diagnosis (TP53, IDH2, ASXL1, DNMT3A). Presence/absence of non-driver mutations, their number (>1), the molecular subgroup (methylation, splicing, chromatin) did not significantly condition OS.

Conclusions: In this work, even if on a small series of patients, we showed that during ruxolitinib about the half of cases developed non-driver mutations, a percentage overlapping to that observed in cases receiving hydroxyurea. Interestingly, ruxolitinib and not hydroxyurea allowed disappearance of mutations in one third of cases.

CO046

DEVELOPMENT OF A COMBINATION STRATEGY BASED ON ER AND OXIDATIVE STRESS TO TARGET ACUTE MYELOID LEUKEMIA

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Introduction: Acute Myeloid Leukemia (AML) is a heterogeneous disease caused by different molecular genetic aberrations. These result in the expression of fusion or mutant proteins that cause impaired differentiation and enhanced proliferation and survival. We previously showed that APL cell lines and primary blasts induced to differentiate by Retinoic Acid (RA) become highly sensitive to amounts of ER stress not detrimental for the same cells in the absence of RA. Furthermore the same cells resulted sensitive to a combination of ER stress inducers with Arsenic Trioxide (ATO) that generates oxidative stress. Importantly we observed that ER stress caused increased amounts of disulphide-bound high molecular weight aggregates of PML-RARα and PML, exacerbating the alteration of cellular proteostasis already generated by induction of ER stress. This observation provides the rationale to translate the findings we observed in APL to other types of AML characterized by fusion or mutant proteins. The presence of mutant proteins that are easily prone to aggregation or mis-folding, because of their mutant structure or because of mis-localization, could render the cells sensitive to levels of ER and oxidative stress that could be recovered in their absence.

Methods: We treated AML cell lines and AML primary leukemic blasts with RA and ER and oxidative stress inducers, evaluating cell proliferation and death, activation of the ER/oxidative stress responses, localization and possible aggregation of the mutant proteins by confocal microscopy, colony forming capacity.

Results: We first tested a panel of AML cell lines characterized by different oncogenic fusion or mutant proteins and we found that ML-2 cells, bearing the MLL-AF6 fusion protein, and MV-4-11 cells, expressing the fusion protein MLL-AF4 and FLT3-ITD are highly sensitive to the combination of sub-lethal amounts of RA, Tm and ATO. In the cells undergoing ER and oxidative stress in combination, we found prolonged activation of the antioxidant response and of the unfolded protein response (UPR), activated by ER stress, as indicated by the expression of HMOX, CHOP, BiP and sXBP1. The antioxidant agent N-acetyl-cystenine and the inhibitor of the UPR player GADD34 determine resistance of the cells to the treatments. Furthermore, an inhibitor of the PERK branch of the UPR dramatically exacerbates the sensitivity to the combination of ER and oxidative stress pointing to this pathway as a possible new therapeutic molecular target. Importantly, the combination of ER and oxidative stress significantly reduces the colony forming capacity of primary leukemic blasts isolated from the bone marrow of FLT3-ITD positive patients.

Conclusions: Altogether our data suggest that the combination of low levels of ER and oxidative stress leads to apoptosis rather than recovery, achieved instead when the same stresses are induced alone.

CO047

IDENTIFICATION OF ALU METHYLATION STATUS BY DROPLET DIGITAL PCR ASSAY IN HEMATOLOGIC MALIGNANCIES

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Introduction: Alu repeats, belonging to the Short Interspersed Repetitive Elements (SINEs) class, contain about 25% of CpG sites in the human genome. They are located in gene-rich regions, so their methylation is an important transcriptional regulation mechanism. Aberrant Alu repeats methylation has been associated with tumor aggressiveness and investigated in some solid tumors, but the global Alu methylation level has not yet been investigated in hematological malignancies. Moreover, today, some of the techniques designed to measure global DNA methylation are focused on the methylation level of specific genomic compartments, including repeat elements. In this work we propose a new method for investigating Alu differential methylation, employing droplet digital PCR (ddPCR) technology, applied in patients affected by chronic lymphocytic leukemia (CLL), myelodysplastic syndromes (MDS) and chronic myelomonocytic leukemia (CMML).

Methods: The study included a total of 45 patients: thirty patients affected by CLL, seven patients with MDS at intermediate/high risk, and nine patients with CMML. The study also involved acute promyelocytic leukemia-derived NB4 cell line, either untreated or treated with azacytidine (AZA) 0.75 μM or decytabine (DEC) 0.75 μM. Four healthy donors (HD) were also included as controls. For each DNA sample two aliquots of 250 ng of gDNA were simultaneously digested (with 1 unit of Alu-in/sensitive isoschizomers either MspI or HpaII) and ligated (to a previously prepared synthetic adaptor) in parallel in two separate tubes. The percentage of methylated consensus Alu sequences was calculated as the ratio between the means of the three values of positive droplets of HpaII (MH) and MspI (MM) diluted digestion-ligation mixtures respectively.

Results: Using our ddPCR assay, we observed a significant decrease of the global Alu methylation level in DNA extracted from NB4 cells treated with DEC, as compared to untreated cells ($p<0.05$), and a slight but not significant decrease with AZA. Moreover, comparing the global Alu methylation levels at diagnosis and after AZA treatment in MDS patients, we observed a statistically significant decrease of Alu sequences methylation after therapy as compared to diagnosis. We also observed a significant decrease of the Alu methylation level in CLL patients compared to HD, and, finally, for CMML patients, a significant decrease of Alu sequences methylation was observed in patients harboring the main SRSF2 gene hotspot.

Conclusions: In our work, we propose a new method to investigate Alu differential methylation based on ddPCR technology. This assay is based on the QUAlu method, but introduces ddPCR as a more sensitive and immediate technique for Alu methylation analysis. To date, this is the first application of ddPCR to study DNA repetitive elements. This approach may be important to profile patients affected by hematologic malignancies for diagnostic/prognostic purpose.

CO048

IDH1 MUTATIONS ASSOCIATE WITH HIGH WT1 EXPRESSION LEVEL IN ACUTE MYELOID LEUKEMIA

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Introduction: Isocitrate dehydrogenase 1/2 (IDH1/2) are frequently mutated genes in Acute Myeloid Leukemia (AML) cases (10-20%). IDH1/2 mutations cause over-production of D2-hydroxyglutarate (2HG), an oncometabolite able to induce DNA hypermethylation, aberrant gene expression and abnormal cell differentiation. The prognostic role of IDH1/2 mutations remains poorly understood but seems to depend on the presence of concomitant molecular lesions. Furthermore, in AMLs, IDH1/2, TET2 and WT1 mutations are mutually exclusive and these three factors have been recently proposed to act on the same pathway The aim of our study is to analyse the molecular and cytogenetic landscape of IDH1/2 mutated AMLs.

Methods: We studied 110 AML retrospective patients, at onset, with a median age of 64 years (range 21-84). The mutational status in R132 of IDH1 and in R140/R172 of IDH2 was analyzed taking advantage of Sanger sequencing. In all cases we characterized karyotype, mutational status of FLT3-ITD/D835, NPM1, CEPBA and expression levels of WT1 and BAALC.

Results: From our study, we identified 15 patients (13.6%) harbouring missense mutations in IDH1 gene (7/15 with R132C, 6/15 with R132H, 1/15 with R132L and 1/15 with R132G) and 13 cases (11.8%) harbouring missense mutations in IDH2 gene (7/13 with R140Q, 1/13 with R140W and 5/13 with R172K). Concurrent IDH1 and IDH2 mutations were not observed. At onset, both IDH1 and IDH2 mutated patients displayed a significant reduced count of white blood cells ($p=0.038$ and $p=0.003$, respectively) as compared to non-mutated IDH1/2. IDH1 mutated (IDH1m) cases showed inverse correlation with complex karyotype ($p=0.035$) as compared to favourable and intermediate cytogenetics and 73.3% of cases had normal karyotype. Most of IDH2m samples harboured intermediate cytogenetic profiles, but no statistical significance was observed. All IDH1m cases displayed WT1 over-expression. Interestingly, IDH1m patients presented significantly higher WT1 expression levels as compared to non–mutated patients ($p=0.007$), showing median WT1 values of 10298 copies and 3369 copies/10000 ABL copies, respectively. As well, a trend of higher WT1 expression was reported in IDH1m as compared to IDH2m cases (median 4376 copies, $p=0.05$). Complete remission, overall survival and disease free survival were not affected by IDH1/2 mutation status.

Conclusions: Overall, our results show that IDH1/2m patients have low white blood cells at onset. Moreover, we confirm that IDH mutations occur mainly in patients classified within intermediate prognostic group For the first time to our knowledge, we report a correlation between IDH mutations and WT1 expression in AML patients. Notably, IDH1 mutated cases significantly associate with higher WT1 levels, supporting data showing that IDH1 and WT1 act on the same pathway. Furthermore, the correlation between IDH1 mutations and WT1 overexpression might be taken into consideration for a better monitoring of AML patients.

Myelodysplastic Syndromes

CO049

ASSOCIATION OF AZACITIDINE AND LENALIDOMIDE (COMBINED VERSUS SEQUENTIAL TREATMENT) IN HIGHER RISK MYELODYSPLASTIC SYNDROMES. UPDATE OF LONG-TERM RESULTS OF A RANDOMIZED PHASE II MULTICENTER STUDY

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Introduction: The association of Azacitidine (AZA) and Lenalidomide (LEN) has proven effective in Myelodysplastic Syndromes (MDS), however the optimum dose and schedule remains unknown. The aim of this study was to evaluate the efficacy and safety of the combination vs the sequential use of AZA and LEN in higher-risk MDS pts. Primary endpoint: ORR, defined as the Rate of Complete Remission (CR), Partial Remission (PR), Marrow Complete Remission (mCR), and Hematological Improvement (HI), following the IWG criteria (Cheson, 2006).

Methods: This is a randomized, phase II, multicenter, open label study, including pts with MDS with IPSS risk High or Intermediate-2, without previous treatment with AZA or LEN. ARM 1 (combined treatment): AZA: 75 mg/m^2/day (days 1-5) I.C. + LEN: 10 mg/day (days 1-21), orally, every 4 weeks. ARM 2 (sequential treatment): AZA: 75 mg/m^2/day (days 1-5) I.C. + LEN: 10 mg/day (days 6-21), orally, every 4 weeks. The induction treatment was planned for 8 cycles. For responder patients the same treatment was continued until disease progression or unacceptable toxicity.

Results: From March 2013, 44 pts (27 males), median age: 72 (48-83 yrs) were enrolled, from 13 hematologic Centers. 21 pts were randomly assigned to ARM 1, and 23 pts to ARM 2. 34/44 pts (77.3%) completed ≥ 6 cycles of treatment, and were considered evaluable for response. Treatment was given for a median of 8.5 (1-52) cycles; in ARM 1: 9 (1-51) cycles; in ARM 2: 8 (1-52) cycles, respectively. Median follow-up: 15 (2-54) months; 47 (37-54) months for survivors. 26/34 pts (ORR: 76.5 %) showed a favourable response to treatment. The Best Response achieved was: CR: 8 pts (23.5%), PR: 1 pt (2.9%), mCR: 3 pts (8.8%), HI: 8 pts (23.5%), mCR+HI: 6 pts (17.6%). The remaining 8 pts showed either Stable Disease (SD) (6 pts, 17.6%) or Disease Progression (DP) (2 pts, 5.9%). Median duration of hematologic response: 10.5 months. 34 pts (77,3%) died , and 17 pts (38.6%) showed progression to AML. Median overall survival (OS): 15 months. No significant differences between the 2 arms were observed, in terms of ORR, CR rate, toxicity, AML incidence and OS, but the median response duration was significantly longer in ARM 2 (18 months) as compared to ARM 1 (6 months) (p=0.0459). At the time of last analysis, 5/44 (11.4%) patients, 1/21 (4.8%) in ARM 1, and 4/23 (17.4%) in ARM 2, were still maintaining the haematological response, and were still in treatment, after 54, 54, 52, 51 and 37 months, and after 52, 51, 33, 48 and 35 cycles of therapy, respectively. The changes observed during treatment in inositide-dependent signalling molecules, mutational status, and microRNA expression profiling were related to response to therapy.

Conclusions: Our results confirm the efficacy of both AZA+LEN treatment regimens in higher-risk MDS pts, in terms of ORR and OS, although patients treated with the sequential regimen showed a significantly longer duration of haematological response.

CO050

GENE MUTATIONS AND MICRORNA SIGNATURE IN MYELODYSPLASTIC SYNDROMES DURING AZACITIDINE AND LENALIDOMIDE THERAPY

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Introduction. Several clinical studies demonstrated the efficacy and safety of combining Azacitidine (AZA) with Lenalidomide (LEN) in MDS, but the molecular implications of this therapy are still under investigation. Here we analyzed the effect of AZA+LEN therapy on MDS, particularly focusing on gene mutations and microRNA profiling.

Methods. The study included 44 high-risk MDS patients (IPSS High or Intermediate-2), who received AZA (75 mg/m2/day, days 1-5, sc) and LEN (10 mg/day, days 1-21 or 6-21, orally) every 4 weeks. Patients were considered clinically evaluable for response after at least 6 cycles of treatment. Response was assessed according to the IWG response criteria. Molecular analyses were performed on mononuclear cells extracted at baseline and after cycles 4th and 8th. Gene mutations were studied by an Illumina TruSight Myeloid Sequencing Panel (54 hematopoietic-specific genes) and an on-demand Ion Torrent gene panel (31 inositide-specific genes), while microRNAs were analyzed by a 4.0 miRNA Affymetrix array.

Results. 34/44 patients were clinically evaluable for response, with an ORR of 76.5% (26/34 cases). For gene mutations, 30 paired samples (pre- and post-treatment) were analyzed: 3 patients showed no mutations neither at baseline nor during therapy, all other patients had at least one mutation. The most frequent mutated genes were ASXL1 (14 cases=47%), TET2 (11 cases=37%), RUNX1 (8 cases=27%) and SRSF2 (5 cases=17%). All patients who showed a decreasing variant allele frequency (VAF) of gene mutations were responders, while all the non-responders had at least one gene mutation and none showed a decreasing VAF. In patients losing the response at the 8th cycle or never responding, a pool of 7 inositide-dependent genes (AKT3, PIK3CD, PIK3CG, MAP2K2, MAP2K1, PLCG2, RPS6KA3) showed acquired mutations during the therapy. Either CEBPA or CBL mutations were associated with a decreased OS (28 vs 14 months and 28 vs 19 months, respectively; p<0.05). Only CEBPA mutation was significantly associated also with the duration of response (27 vs 6 months; p<0.05), while CBL mutation was close to significant (27 vs 9 months, p=0.08). For microRNA profiling, at the 4th cycle we had a cluster of 43 microRNAs up- or down-regulated, as compared with baseline, and at the 8th cycle the cluster included 19 microRNAs. Interestingly, in patients losing the response at the 8th cycle, 8 microRNAs showed a statistically significant difference, with 5 up-regulated (e.g. miR-4767) and 3 down-regulated microRNAs (e.g. mir-424).

Conclusions. Our results show that AZA+LEN therapy in high-risk MDS patients can induce a favourable clinical response, and that the molecular mutation/microRNA profiling could be important to better disclose the effect of the therapy, particularly the loss of response. Further studies are needed to confirm all these data, and possibly better understand the role of nuclear inositide signalling in MDS.

CO051

PROGNOSTIC MARKERS OF RESPONSE AND SURVIVAL IN HR-MDS TREATED WITH AZACITIDINE AS BRIDGE TO ALLOGENEIC STEM CELL TRANSPLANTATION

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Introduction: Hypomethylating treatment, in particular azacitidine (AZA), is the standard of care in patients with higher-risk MDS. However, HSCT remains the only curative option in eligible patients. Our aim was to evaluate the association between specific somatic mutations, response to AZA and HSCT outcome in HR-MDS and LBC-AML.

Methods: Sixty-five patients (53 de novo HR-MDS and 12 LBC AML) with median age 59 years (range 21-66 years) enrolled in BMT-AZA multicenter trial, were studied. All patients were homogeneously treated with a standard AZA regimen, for at least 4 cycles, followed by HSCT in 44 patients. Patients were considered responders to AZA, if they achieved complete remission (CR), partial remission (PR) or haematological improvement (HI). Patients exhibiting stable disease (SD) or progressive disease (PD) were considered resistant. Ultra-deep NGS (Myeloid Solution panel by Sophia Genetics on an Illumina platform) was performed on DNA extracted from BM mononuclear cell samples obtained before starting AZA treatment. Analysis of variant allele frequency (VAF) was performed using a standardized approach and the SOPHiA DDM® software. Changes in mutation burden were studied in selected cases after 4 cycles of AZA, using specific pyrosequencing assays.

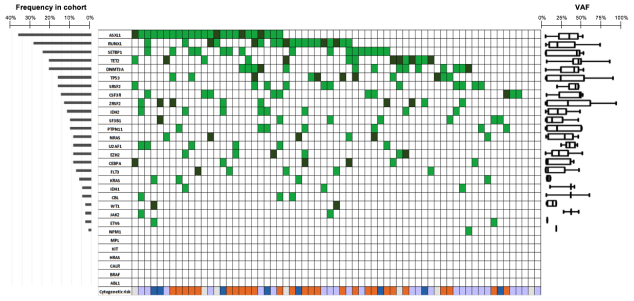

Figure 1. Distribution, frequency and variant allele frequency (VAF) of mutations in the study cohort. Each column represents a single patient. Light green boxes represent a mutation, dark green boxes represent the presence of >1 mutations in the same gene, whereas empty boxes indicate wild type genes. According to IPSS, lilac boxes represent patients with good cytogenetic risk, blue boxes represent patients with intermediate cytogenetic risk and orange boxes represent patients with poor cytogenetic risk. Grey boxes indicate patients with cytogenetic profile not available.

Results: At diagnosis, we identified at least 1 mutation, at VAF greater than 1%, in 62 out of 65 patients (95.4%). The most commonly mutated genes were: ASXL1 (37%), RUNX1 (29%), SETBP1 (25%), DNMT3A (21%), and TET2 (21%). Thirty-one of 62 patients (50%) had more than 1 mutation in the same gene (Figure 1). Univariate analyses revealed that mutations in DNMT3A (VAF greater than 10%) were associated with lower response rate (p=0.051). In particular, DNMT3A mutations localized in the methyltransferase domain were found in 11 of 65 patients (16.9%): only 1 patient in this subgroup achieved HI, whereas the remaining 10 patients were unresponsive. The R882 mutation, present in 50% of DNMT3A-mutated patients (7 of 14), was identified only in unresponsive patients. Similarly, 9 of 65 SETBP1 mutated patients (13.8%) had mutations in the SET-binding domain or in the SKI homologous region (HR) and only one of them achieved HI, whereas 8 patients were unresponsive to AZA treatment (p=0.0350). Allelic frequency of most mutations did not change upon AZA treatment, while in six AZA-responsive patients (3 CR, 2 PR and 1HI) a reduction of TP53-mutations was observed after 4 cycles. However, at the multivariable analysis, TP53 mutations retained its unfavourable prognostic significance in terms of either PFS or OS (p=0.0013 and p=0.0008, respectively), together with AZA response (p=0.0003 and 0.0068, for PFS and OS). When restricting the analysis to patients who underwent HSCT, TP53 confirmed its negative prognostic role.

Conclusions: Our data show that mutational screening of HR-MDS and LBC-AML using a standardized NGS approach may predict response to AZA treatment and survival after HSCT.

CO052

CIRCULATING INFLAMMA MI-RNAS UPREGULATION IN MYELODYSPLASTIC SYNDROMES

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Introduction: Myelodysplastic Syndromes (MDS) are a heterogeneous group of clonal stem cell disorders affecting mainly the elderly and characterized by ineffective haemopoiesis, peripheral blood cytopenias and risk of developing acute myeloid leukemia.Immune system dysregulation may represent a primary pathophysiologic abnormality and a key driver of the pathological evolution of MDS and it looks like chronic inflammation role is essential in MDS pathogenesis and progression: there is growing evidence implicating inflammation-related changes, inhibitory cytokines and increased intramedullary apoptosis as contributors to ineffective hematopoiesis. MicroRNAs (miRNAs) are a broad class of small, non-coding RNAs involved in the modulation of gene expression, either silencing or activating transcription via sequence-specific DNA/RNA binding. Such mediators of epigenetic information have been extensively investigated, in recent years, as potential biomarkers of age related desease. Recently some miRNAs, such as miR-146, miR-155, and miR-21, were found to be associated with inflammation and senescence. In this study we evaluated the expression of 15 miRNA which have been demonstrated to be dysregulated during ageing and in inflammatory conditions

Methods: In this study, we evaluated: miR-17; miR-9; miR-22; miR-152; miR-335; miR-19b; miR-20a; miR-34a; miR-146a; miR-181a; miR-9; miR-21; miR-126; miR-29a; miR-155. Total RNA was isolated from the serum of 80 MDS patients and 80 donors, matched for age and sex, and a quantitative analysis of the circulating miRNA was performed by Real Time PCR.

Results: Among 15 miRNA studied, the levels of miR-9, miR-17, miR-22, miR-34a, miR-152, and miR-335 were not detectable both in patients and donors. MiR-126, miR29a, miR20a, miR181a, miR21, miR155 and miR146a show a statistically significant differential expression in MDS with respect to controls. In particular, we observed an upregulated expression of miR126, 20a, 21 and 146a, in LR-IPSS patients,

and of miR155 in HR-IPSS patients.

Conclusions: In this work it has been possible to evaluate circulating miRNAs levels in MDS. Data showed an upregulation of these "inflamma-miRNAs" in particular in LR MDS, respect to controls and this could be the key regulators in the pathogenesis of ineffective erythropoiesis and anemia and also in the increased proliferation pressure on progenitor cells and subsequent genetic instability and somatic mutation during the course of the desease.

CO053

NEXT GENERATION SEQUENCING OF GENES FREQUENTLY MUTATED IN MYELOID MALIGNANCIES MAY HELP TO PREDICT HEMATOLOGIC RESPONSE TO THE IRON-CHELATOR DEFERASIROX IN MDS PATIENTS

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Introduction: Red blood cell (RBC) transfusions are an important component of supportive care in patents with lower-risk MDS. Iron accumulation has been recognized as a deleterious prognostic factor in myelodisplastic syndromes (MDS), and iron-chelating therapy is indicated in patients with high ferritin levels and/or a history of red-blood-cell transfusions. Iron chelation has been associated to survival prolongation and hematologic improvement in 10-20% of MDS cases. Somatic mutations are one of the major factors driving the pathogenesis of MDS.

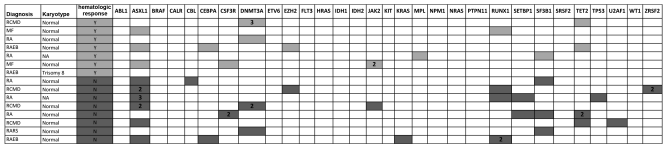
Methods: We studied by NGS the mutational profile of critical genes in 19 patients who achieved hematologic improvement (HI) during Deferasirox (DFX) treatment (31.7%) and 41 patients who did not (68.3%). Thirty genes, frequently mutated in myeloid malignancies, were screened for somatic mutations in 15 patients (7 responsive and 8 resistant) using NGS and the commercial Myeloid Solution by SOPHiA GENETICS (SOPHiA GENETICS, Saint-Sulpice, Switzerland). Fourty-five patients were then screened for mutations in the hotspot regions of spliceosome machinery enzymes (SF3B1, SRSF2, U2AF1) and epigenetic regulators (IDH1, IDH2 and DNMT3A), using Sanger sequencing.

Results: Treatment with DFX lead to a significant decrease in ferritin levels after 6 months when compared to basal levels (1625 vs 2209 ng/ml, p=0.0011). However, none of the clinical factors analyzed was associated to HR to DFX. We identified 53 mutations in 14 of 15 patients (93.3%), with a median of 3.53 (range, 0-6) mutations per patient. The most commonly mutated genes were: ASXL1 in 9 of 15 patients (60%), and RUNX1, DNMT3A, SF3B1 and TET2 in 4 of 15 patients (27% each) (Figure). None of the single gene mutation was predictive of HR to DFX, but mutation number was lower in DFX responders, as compared to resistant patients (mean 2.4 vs 4.5 mutations/patient, respectively; p=0.0232). In the additional cohort of 45 patients screened for mutations in the spliceosome machinery enzymes and epigenetic regulators, SF3B1 was the most commonly mutated gene (19/45 patients, 42.2%), but there was no association between mutations and HR (14 mutated in 33 resistant patients (42%), versus 5 of 12 responsive patients, (42%). As expected, SF3B1 mutations were prevalent in refractory anemia with ring sideroblast (8 out of 9 patients, 89%), but HR rate to DFX in this patient subgroup was similar to that of all other patients (3 out of 9, 33% versus 16 out of 51, 31%). SF3B1 mutations were associated to higher platelet counts and lower bone marrow blast proportion at initial MDS diagnosis, as compared to wild type patients (p=0.0092, and p=0.0350, respectively).

Conclusions: Iron accumulation in ringed sideroblasts and mutations of the spliceosome machinery are not predictive of HR after iron-chelating treatment, whereas mutational screening performed by NGS may have a role in predicting HR to Deferasirox.

Figure 1.

Distribution of mutations identified in MDS patients treated with the iron-chelator Deferasirox. Colored boxes represent mutated patients (light grey for responders and dark grey for resistant), whereas empty boxes indicate wild-type patients. The numbers in boxes indicates the number of mutations identified in the same gene. MDS subtypes, including RA (refractory anemia), RARS (refractory anemia with ring sideroblasts), RCMD (refractory cytopenia with multilineage dysplasia), RAEB (refractory anemia with excess of blasts) are reported. The case series also included one patient with PMF (primary myelofibrosis).



CO054

VARIABILITY OF ENDOGENOUS ERYTHROPOIETIN LEVELS IN PATIENTS WITH LOWER-RISK MYELODYSPLASTIC SYNDROMES

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Introduction: Myelodysplastic syndromes (MDS) are a heterogeneous group of myeloid diseases characterized by ineffective hematopoiesis, peripheral blood (PB) cytopenia and a tendency to evolve into acute myeloid leukemia (AML). Patients with lower-risk MDS (LR-MDS) are frequently affected by severe anemia due to ineffective hematopoiesis. Current guidelines prescribe treatment with erythropoiesis stimulating agents (ESA) in these patients provided that hemoglobin (HB) levels are lower than 10g/dl and serum eryhtropoietin (EPO) lower than 500 mIU/ml. We investigated the distribution of endogenous EPO levels in a cohort of 296 LR-MDS, in order to dissect the intrinsic variability of this parameter and its pathophysiological impact on MDS erythropoiesis.

Methods: Hematological and clinical characteristics of 296 patients with LR-MDS enrolled in the GROM-L (Gruppo Romano-Laziale sindromi Mielodisplastiche) registry were analyzed. Using linear regression models, we correlated baseline EPO levels to standard clinical parameters at diagnosis, including age, sex, weight, hematological parameters (Hemoglobin, Hb, EPO, ferritin, reticulocytes, LDH, MCV, creatinine), bone marrow (BM) dysplastic changes (defined by the presence of >10% morphological abnormalities in erythroid, granuloblastic or megakaryocyte lineage). Data on erythroid response at 6 months was also available.

Results: As expected, there was an indirect correlation between lower Epo levels at diagnosis and erythroid response at 6 months after ESA treatment start (p<.0001). Using Epo levels as continuous variable, we found a positive correlation between EPO and mean corpuscolar volume (MCV, p= 0.0175) possibly explained by alterations in terminal eryhtroblasts differentiation. On the other hand, there was a negative cor-

relation between levels of endogenous EPO, and of Hb, proportion of erythroblast in BM, creatinine, and LDH (p= 0.0017, p= 0.0065, p=0.0088, and p=0.0337, respectively). These correlations were confirmed by the multivariable analysis (Table 1). The direct association between higher Epo levels and increased eryhtroblast proportion in BM may reflect the attempt to compensate ineffective erythropoiesis by increased Epo production, while the inverse correlation between EPO levels and creatinine abnormality is a known mechanism associated to "oxygen sensing" alterations in elderly patients with renal impairment. Of note, the inverse correlation between LDH and EPO may underline the severity of ineffective erythropoiesis, possibly explained by abnormal accumulation of zinc protoporyphyrin concomitant with heme synthesis.

Conclusions: We conclude that the complex pathophysiology of anemia in LR-MDS may be partially explained by EPO signaling impairment. Increased EPO levels may be an attempt to overcome the severity of ineffective erythropoiesis, a well-known hallmark of MDS.

Table 1. Multivariable analysis: linear Regression Model for EPO levels (considered as a continuous variable).

Parameter	Estimate	95% Lower CI	95% Upper CI	p value
Hb	-37.49	-54.23	-20.75	<.0001
MCV	2.43	0.18	4.68	0.0366
Erythroblasts	-2.94	-4.88	-1	0.0032
Creatinine:Abn vs norm	-62.1	-122.47	-1.73	0.0449
LDH	-0.25	-0.41	-0.09	0.0011

CO055

ALPHA LIPOIC ACID SHOWS ANTIOXIDANT AND CHELATING PROPERTIES AGAINST THE TOXIC EFFECTS INDUCED BY IRON OVERLOAD TREATMENT

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Background: Secondary iron overload syndromes are due to hematological diseases such as thalassemias, transfusion-dependent anemias and myelodysplastic disorders. Several organs are affected by iron overload including liver, heart and endocrine glands. A comprehensive approach including tailored transfusion protocols, monitoring and assessment of total body iron levels and iron chelation is currently the mainstay in hindering iron overload. Numerous studies suggest that iron chelation improves survival of trasfusion-dependent patients. The aim of the present study was to investigate whether alpha- Lipoic Acid (ALA), a naturally occurring substance, reduces cellular damage induced by iron overload focusing on its antioxidant and chelating properties *in vitro*, on a stromal cell line and *in vivo*, using an adult zebrafish model.

Methods: HS-5 cell line (stromal cells) was treated with Ferric Citrate Ammonium (FAC) 25µg/mL alone and in combination with ALA 20 µg/ml. Oxidative stress was evaluated by flow-cytometry, western blot, immunofluorescence, autophagy induction by AVO-test and Fe3+ stores using the Perls staining. Concerning *in vivo* model, adults zebrafish were treated with FAC 120µg/ml alone and in combination with ALA 20 µg/ml up to 48h. Specimens were fixed in formaldehyde 4% and Hematoxilin-Eosin and Perls staining were performed. Gene expression analysis of oxidative stress markers (HMOX1b, mtSOD and FPN1) was carried out by real time quantitative RT-PCR.

Results: HS5 cells co-treatment with FAC plus ALA was able to reduce the oxidative stress, measured by all the different methods, induced by FAC alone ($p<0.001$). Iron overload induced the upregulation of oxidative stress marker genes and proteins HO-1 (heme oxygenase1) and SOD (superoxide dismutase), after 24h of FAC treatment. Interestingly, ALA co-treatment improves both mitochondrial integrity, increasing EF-Tu protein levels, and cellular homeostasis, decreasing the autophagolysosomes formation ($p<0.001$) compared to iron alone treatment. In addition, co-treatment was able to induce glutathione synthesis ($p<0.0001$) and to restore the mitochondrial membrane potential ($p<0.001$) after mitochondrial damage induced by iron accumulation ($p<0.001$). Looking at *in vivo* results, ALA protects zebrafish intestine, liver, heart and gills from iron overload showing its ability to prevent histological alterations and to reduce both the oxidative stress expression markers (HMOX1b, mtSOD; $p<0.001$) and Ferroportin1 (FPN1; $p<0.001$).

Conclusions: Our data suggest that ALA protects against iron overload mediated damages through suppression of oxidative stress induced by iron overload, reduction of cell autophagy, restoring mitochondrial integrity and preventing iron overolad-induced organ damages, both *in vitro* and *in vivo* models. Our findings back up the novel idea that ALA supplementation could be of help in countering secondary iron overload related diseases.

CO056

TARGETED SEQUENCING ANALYSIS OF COMMONLY MUTATED GENES IN MYELODYSPLASTIC SYNDROMES USING NGS: IMPACT AND CLINICAL IMPLICATIONS IN A SINGLE CENTER

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Introduction: The pathogenetic role of mutations in myelodysplastic syndromes (MDS) is presently investigated, and important clinical implications of these mutations are apparent. Clinical guidelines suggest including mutation evaluation in current practice.

Methods: We sequenced a panel of 22 genes using Haloplex system and the Miseq platform to assess the mutational landscape of MDS patients. We evaluated 98 sequential MDS patients diagnosed in our center, with prevalence of younger ones: median age of 67 years (range, 23–88), 61 were male, 62% belonging to lower IPSS-R.

Results: The mean depth of the targeted resequencing was 3441X across the entire cohort. A total of 140 SNV and indels were called in 11 genes as high-probability somatic changes. At least one genomic alteration was observed in 89% of patients. The most frequently mutated genes were TET2 (26%), SF3B1 (24%), DNMT3A (22%), SRSF2 (21%), ASXL1 (21%), RUNX1 (9%), TP53 (7%), U2AF1, IDH1, CBL and SETBP1 were mutated in < 5% of the patients. The mean follow up of our cohort was 25 months (range, 4–94). The univariate analysis showed that only age (< 70 years >) (P=0.04), WHO classification (low risk vs high risk (RAEB-1/RAEB-2), P=0.001) and IPSS-R (P=0.05) influenced significantly OS. Regarding somatic mutations, only RUNX1 mutations were associated with a shorter OS (49 months (WT) vs 12 months (mut); P= 0.001). We also analysed the impact of mutations in patients with normal karyotype (n = 73) where DNMT3A mutations conferred shorter OS (49 months vs 23 months; P=0.02). The median number of mutations was 2 (0-4) in this subgroup of pts. A trend to shorter OS was observed in patients with higher number of mutations (2 -4) (P= 0.07). However, a significant difference in OS was observed when two groups: pts with 0-1 vs ≥2 mutations were compared: OS of pts with ≥2 mutations was 25 months vs 49 months; P= 0.003), consistent with what shown previously.

Discussion: Our experience confirms that the presence of even isolated RUNX1 mutations, although rare, identify patients with shorter survival. Patients with normal karyotype although belonging frequently to IPSS-R lower risk category, when carrying DNMT3A mutations or >2 somatic mutations have a significantly shorter survival than predicted by IPSS-R. Our observations, although carried out in a limited group of cases, strongly confirm the prognostic importance of mutations, and support the implementation of NGS analysis of somatic mutation, especially for MDS patients without cytogenetic abnormalities.

Myeloproliferative Disorders and Chronic Myeloid Leukemia

CO057

LOSS OF FUNCTION OF THE SETD2 TUMOR SUPPRESSOR IS A RECURRENT EVENT IN ADVANCED-PHASE CHRONIC MYELOID LEUKEMIA, IS DUE TO POST-TRANSLATIONAL MECHANISMS AND FOSTERS GENETIC INSTABILITY

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Introduction: Inactivating mutations in the SETD2 tumor suppressor occur in solid tumors and acute leukemias. SETD2 trimethylates histone H3 Lysine 36 (H3K36Me3) and plays a key role in transcription and splicing, homologous recombination (HR), mismatch repair. In Systemic Mastocytosis, we have demonstrated that SETD2 loss of function may occur at the post-translational level.

Methods: Western Blotting (WB) was used to assess H3K36Me3 levels and to screen for SETD2 protein expression in a cohort of 80 advanced-phase CML patients (pts). SETD2 mutations and transcript levels were investigated by NGS and real time PCR. Co-immunoprecipitation (co-IP) was used to study protein interactions. Immunofluorescence (IF) with an anti-phospho-histone 2A.X (γH2AX) and an anti-Rad51 antibody was used to evaluate DNA damage and HR repair. Apoptosis and clonogenic assays were performed to test sensitivity to proteasome and MDM2 inhibitors.

Results: Reduced or null SETD2 and H3K36Me3 were detected in 86% of pts as compared to a pool of healthy donors and to CP pts at diagnosis who achieved optimal responses to TKIs, but neither mutations/deletions nor mRNA down-regulation were found. Proteasome inhibition in primary cells from pts with undetectable SETD2 restored H3K36Me3 and led to accumulation of hyper-ubiquitinated SETD2. Moreover, it induced apoptosis and reduced clonogenic growth. In K562 cells (SETD2/H3K36Me3low), co-IP performed before and after proteasome inhibition showed that SETD2 interacts with MDM2 and, as a result, it is hyper-ubiquitinated. MDM2 inhibition by SP-141 resulted in cytostatic effects and rescued SETD2 expression and activity. The latter was also achieved by siRNA-mediated silencing of MDM2, suggesting that MDM2 is implicated in SETD2 reduced stability. Co-IP also showed that SETD2 interacts with Aurora Kinase A (AKA) a Ser-Thr kinase frequently overexpressed in CML. We found that AKA phosphorylates SETD2, and both pharmacological inhibition by Danusertib and siRNA-mediated silencing rescued SETD2 expression and activity. To investigate whether SETD2/H3K36Me3 loss contribute to genetic instability, LAMA 84 (SETD2/H3K36Me3high) and K562 (SETD2/H3K36Me3low) cells were studied by WB and IF to assess γH2AX and Rad51 in steady state and after sub-lethal DNA damage by UV exposure. The same studies were performed after siRNA silencing of SETD2 for 3 months. Cells with low or silenced SETD2 had significantly higher levels of γH2AX and were unable to induce HR repair. Clonogenic assays in LAMA 84 before and after SETD2 silencing suggested that reduction of clonogenic growth after proteasomal or MDM2 inhibition is indeed SETD2-dependent.

Conclusions: Phosphorylation by AKA and ubiquitination by MDM2 contribute to SETD2 non-genomic loss of function in advanced-phase CML. Loss of SETD2/H3K36Me3 result in increased DNA damage and impaired HR repair. Restoring physiological H3K36Me3 levels may help improve the outcome of this critical subset of pts.

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CO058

A NOVEL GERMLINE MUTATION IN THE 3' UNTRANSLATED REGION OF CALRETICULIN GENE INDUCES JAK/STAT SIGNALING ACTIVATION AND ERYTHROCYTOSIS

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Introduction: The driver mutations of MPNs, occurring on JAK2, MPL and Calreticulin (CALR) genes, cause a deregulation of JAK/STAT signalling. CARL mutants, associated almost exclusively with ET and PMF, activate JAK/STAT by interacting with TPO-receptor. CALR mutations generally arise from a +1 frameshift that generates a common novel peptide lacking KDEL ER-retrieval signal, converting the first 31 bases of CALR 3'UTR into coding sequence. However, the hematopoietic and regulative functions of this 3'UTR region are still unknown. Here we show the clinical and biological consequences of a novel deletion of CALR 3'UTR, designated c.1254+10_+33del24. This mutation occurred 10 bp downstream the CALR stop codon, not altering the coding sequence. Interestingly, these patients were diagnosed with JAK2V617F-negative PV.

Methods: CALR mutations and mutant allele burden were detected by PCR screening, Sanger sequencing and fragment analysis on DNA from granulocytes, CD34+-hematopoietic progenitor cells and saliva epithelium. CALR mRNA was measured by quantitative RT-PCR. CALR, STAT3/5 and p-STAT3/5 protein levels were assessed by immunoblotting. The colony assays were performed using PB mononuclear cells isolated by Ficoll. Cell immunophenotype was assessed by FACS. Deletion of 3'UTR sequences were obtained by CRISPR-CAS9 technology.

Results: CALR 3'UTR deletion c.1254+10_+33del24 was detected in granulocytes, CD34+-HPCs and oral epithelial cells from saliva of two sibling diagnosed as JAK2V617F-negative PV patients, suggesting that the mutation is germline. This deletion is located within an evolutionarily conserved region at nucleotides 10-33 of 3'UTR, thus not altering the KDEL domain. In all samples, the mutant allele burden was >50%. Both siblings showed an enhanced erythropoiesis, also assessed by BFU-E growth at low erythropoietin conditions. Moreover, increased CALR mRNA/protein levels and aberrant activation of the JAK/STAT pathway were detected in PB samples from these patients and other MPNs affected by canonical CALR mutations. Importantly, the removal of region 10-33 of 3'UTR by CRISPR-CAS9 technology in K562 cells induced an overproduction of differentiated erythroid cells, documented by the reduction of c-kit receptor (c-kitR/CD117+) and the rise of CD235a+ erythroid markers, by increasing the levels of CALR and p-STAT5.

Conclusions: CALR mutations in MPNs have hitherto been observed only in ET and PMF, where the mutation generates a novel CALR peptide sequence. Here we report that the mutation c.1254+10_+33del24, deleting 24 bp of CALR 3'UTR, is functionally associated with erythrocytosis and correlates with increased CALR expression and JAK/STAT signalling activation. Our results suggest that CALR 3'UTR functional impairment reprograms myeloid progenitors towards erythropoiesis and reproduces a PV phenotype, possibly operating on RNA intracellular localization, microRNA binding, translational efficiency or mRNA editing.

CO059

SETD2 NON-GENOMIC LOSS OF FUNCTION IN ADVANCED SYSTEMIC MASTOCYTOSIS (SM): PATHOGENETIC AND THERAPEUTIC IMPLICATIONS

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Introduction: We have recently found that the HMC-1.1 and -1.2 MCL cell lines and advanced SM patients (pts) display histone H3 Lys36 trimethylation (H3K36Me3) deficiency as a result of non-genomic loss of function of the SETD2 methyltransferase. SETD2 plays a key role in the control of transcription and splicing fidelity and homologous recombination (HR). Inhibition of proteasome-mediated degradation by bortezomib restored SETD2 protein expression and H3K36Me3.

Methods and Results: To investigate whether increased DNA damage and reduced HR proficiency can be observed in SETD2/H3K36Me3-deficient SM, we used western blotting (WB) and immunofluorescence (IF) to assess phosphorylated histone 2A.X (γH2AX) and Rad51. Compared to cells from healthy controls, SETD2- and H3K36Me3-deficient cell lines and pts had significantly higher levels of γH2AX and lower levels of Rad51. RNA-seq in SETD2-deficient pts showed evidence of transcription and splicing defects like transcription-induced chimeras and intron retention not observed in healthy donors. We found that SETD2 co-immunoprecipitates (co-IP) with p53 in HMC-1.1 and -1.2 cells. Rescuing the interaction between SETD2 and p53 with bortezomib stabilized p53 and upregulated the expression levels of p53 targets including p21, p27, Bax and Gadd45a. This restored inhibition of cell proliferation, block of the transition towards cell cycle checkpoints and consequent activation of apoptosis. The ubiquitin E3 ligase MDM2 was also found to complex with SETD2 after proteasomal inhibition. Treatment with the MDM2 inhibitor SP-141 rescued SETD2 expression and H3K36Me3, suggesting that MDM2 may play a role in SETD2 degradation in ASM and MCL. Moreover, SP-141 treatment of HMC-1 cells at micromolar doses induced cytostatic but not cytotoxic effects as shown by cell growth curves. Clonogenic assays supported the cytostatic effects of SP141 in HMC-1.1 and -1.2 cells. siRNA-mediated knock-down of MDM2 also rescued SETD2 expression and activity, supporting the hypothesis that SETD2 hyper-ubiquitination by MDM2 plays a role in SETD2 reduced stability and proteasomal degradation. Co-IP also showed that SETD2 interacts with Aurora Kinase A (AKA), that is over-expressed in advanced SM. We found that AKA phosphorylates SETD2 and this phosphorylation may be involved in SETD2 loss of function. In fact, both inhibition by Danusertib and siRNA-mediated silencing rescued SETD2 expression and activity.

Conclusions: AKA triggered MDM2-mediated ubiquitination contributes to SETD2 non-genomic loss of function in ASM and MCL. Loss of SETD2 and H3K36Me3 is associated with increased DNA damage and transcription and splicing defects in ASM and MCL pts and is likely to afford an alternative mechanism for the inactivation of the p53-mediated checkpoint without the need for TP53 gene mutations. Inhibiting AKA or MDM2 activity or proteasome-mediated degradation are promising therapeutic strategies. Supported by AIRC (project 16996) and AIL.

CO060

GENE EXPRESSION PROFILE IDENTIFY PATIENTS WITH DIFFERENT PROGNOSIS IN PRIMARY AND SECONDARY MYELOFIBROSIS

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Introduction: Classic Philadelphia-negative myeloproliferative neoplasms include three distinct clinical entities: polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF). PV and ET can evolve to secondary myelofibrosis (SMF) giving rise to post-PV (PPV) and post-ET (PET) myelofibrosis (MF). PMF and SMF patients are currently managed in the same way and prediction of survival is based on the same prognostication models, even if it has been demonstrated that they can't accurately distinguish different risk categories in SMF. In order to improve risk class definition in MF, we profiled CD34+ cells from PMF and SMF patients. This approach has already been applied successfully in several other hematological malignancies including acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS) allowing the identification of patients' subgroups characterized by poor clinical outcome.

Methods: Eighty-six MF patients were included in this study: 4 prefibrotic/early PMF, 43 overt PMF, 29 PET and 10 PPV. MF CD34+ cells were purified from peripheral blood and total cellular RNA was extracted. Gene expression profile (GEP) was performed using the Affymetrix platform. In order to identify genes whose expression is related to survival in MF patients, we performed a Cox regression analysis by means of Partek GS Software. Moreover, the same analysis was carried out on miRNA expression profile (miEP), performed on the same samples using the Affymetrix platform.

Results: Cox regression analysis led to the identification of a gene set which could discriminate high-risk (HR) from low-risk (LR) MF patients. As shown in Figure 1, hierarchical clustering of samples according to the expression of survival related genes separated MF patients into two subgroups with a different outcome, indeed HR patients have a lower overall survival when compared with LR ones. Interestingly, 14 out of 15 patients (93%) in the HR subgroup has died. The analysis of genes identified by Cox regression revealed that many of them are involved in several pathways already described as deregulated in myeloid malignancies, such as PI3K/AKT, mTOR and ERK/MAPK. We also identified 5 miRNAs (i.e. miR-92a, miR-127, miR-320, miR-324 and

miR-155) whose differential expression is associated to clinical outcome in MF patients.

Conclusions: As a whole, our results suggest that GEP and miEP are useful tools for risk prediction in PMF and SMF, since it can improve the identification of patients' subgroups characterized by a poor prognosis. The expression of these survival related genes and miRNAs will be monitored in granulocytes from an independent cohort of patients in order to validate the clinical relevance of this prognostication model.

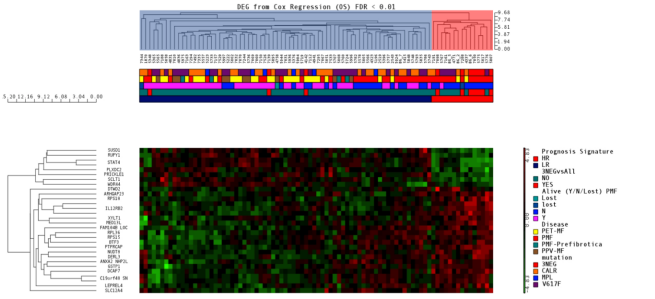

Figure 1.

CO061

NGS-BASED PROSPECTIVE ASSESSMENT OF LOW BURDEN MUTATION DYNAMICS IN CHRONIC MYELOID LEUKEMIA PATIENTS WITH FAILURE AND WARNING RESPONSES: THE 'NEXT-IN-CML' STUDY

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BCR-ABL1 mutations are not the only mechanism of resistant to tyrosine kinase inhibitors (TKIs) acting in chronic myeloid leukemia (CML) patients (pts) and not even the most frequent - yet they are the only actionable one. How frequently low-burden mutations can be detected by Next Generation Sequencing (NGS) in pts with Failure or Warning response to TKIs and which role they play in these pts remain to be addressed prospectively in large series of unselected pts. Moreover, the implementation of routine, NGS-based BCR-ABL1 mutation screening in a molecular diagnostic lab network has never been attempted. To assess the feasibility, performance, cost, turnaround times and clinical utility of NGS, we have conducted a multicenter prospective study: 'Next-in-CML'. The first phase of the study was aimed to 1) create a network of 4 reference labs sharing a common protocol and an optimized pipeline of analysis; 2) perform a control round to assess the accuracy and inter-lab reproducibility. After this phase was successfully completed, 3% was taken as lower detection limit. The second phase of the study was aimed to apply NGS in parallel with Sanger seq in a consecutive series of prospectively collected samples from 214 CML pts with Failure or Warning treated at 39 Hematology Centers. Pts positive for any point mutation (the 35bp insertion was excluded) were 42 (20%) by Sanger seq and 94 (44%) by NGS. Low-burden mutations detectable by NGS were found in 52 pts negative for mutations by Sanger seq; in addition, 24 pts positive for mutations by Sanger seq were found to carry additional low-burden mutations by NGS, for a total of 76 pts harboring low-burden mutations. Eight pts had a low-burden T315I and 43 pts had other IM/DAS/NIL/BOS resistant mutations: overall, 51 (24%) pts had relevant TKI-resistant low-burden mutations missed by Sanger seq. The remaining 25 pts had only mutations with unknown resistance profile. Follow-up samples and clinical information of patients harboring low-burden mutations showed that TKI-resistant mutations remain consistently detectable and tend to increase in burden at subsequent timepoints whenever treatment is not changed or is changed to a TKI non active against those mutations. Longitudinal follow-up of pts with low-burden mutations of unknown resistance profile showed that some mutations may persist for some time while others may disappear at the subsequent timepoint but do not necessarily require treatment intervention to be eliminated. Representative mutation dynamics will be shown. In conclusion: the 'Next-in-CML' study shows that NGS of BCR-ABL1 is feasible, robust and reproducible and suggests that low-burden (≥3%) TKI-resistant mutations are sufficient to drive clonal expansion. Additional studies will be needed to understand the clinical significance, if any, of the mutations with unknown resistance profile.

CO063

FUNCTIONAL CHARACTERIZATION OF THE -2518 A/G SINGLE NUCLEOTIDE POLYMORPHISM OF MCP-1 IN MYELOFIBROSIS

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Introduction: Host genetic variations have an essential role in the mutational landscape of Philadelphia-negative MPN, with JAK2 46/1 and TERT rs2736100 polymorphisms predisposing to disease onset (Trifa AP *et al.*, Am J Hematol. 2018, Tapper W *et al.*, Nat Commun. 2015). However, the contribution of inherited factors in disease phenotype and evolution is poorly characterized. In MPNs, chronic inflammation triggers neoplastic transformation and catalyzes clonal evolution toward end-stage disease (Hasselbalch C, Blood 2012). We recently demonstrated that the -2518 A/G SNP of the Monocyte Chemoattractant Protein-1 (MCP-1, rs1024611) is an inherited host genetic factor associated with secondary myelofibrosis (sMF) and a biomarker of disease severity in MF (Masselli E *et al.*, Leukemia 2018). Here we aimed to characterize MCP-1 expression in MF according to patients' genotype, and the potential cellular source(s) of this chemokine.

Methods: Fifteen therapy-naïve MF patients were recruited for this study. Four healthy subjects and four apheresis bags were utilized as controls (CTRL) in the experiments. MF were stratified according to their rs1024611 genotype in A/A (wild type), A/G and G/G (polymorphic). Peripheral blood mononuclear cells (MNCs) were isolated by

Ficoll-Hypaque gradient, in part pelleted (resting, T0) and in part seeded in RPMI-1640 medium and activated with 1.1 ng/ml of IL-1β for 20 hrs (T1). T0 and T1 cells were processed for RNA extraction. CD34+-cells were purified from MF peripheral blood and from apheresis bags by immunomagnetic selection (Miltenyi Biotech) and differentiated toward the MK lineage as described in Masselli E *et al.*, Leukemia 2015. MK differentiation was assessed by flow cytometric expression of CD41. MKs were then processed for RNA extraction. MCP-1 expression was evaluated by real-time PCR.

Results: We demonstrated that MF-MNCs significantly over-expressed MCP-1 as compared to CTRL-MNCs (Panel A) at basal state. When MF-MNCs were stimulated ex-vivo with IL-1β, we observed a dose-dependent effect of the -2518 A/G SNP on MCP-1 expression, with polymorphic patients displaying a >100-times higher fold-increase (T1 vs. T0) in MCP-1 expression as compared to A/A (Panel B). MF-MKs also showed a significantly higher expression of MCP-1 as compared to CTRL (Panel C). Finally, MF-CD34+-cells from A/G+G/G patients displayed impaired MK differentiation compared to A/A, as indicated by a significantly lower number of CD41+-cells obtained in culture (Panel D).

Conclusions: Our data show that circulating MNCs and CD34+-derived MKs are a major source of MCP-1 in MF. Polymorphic MF patients, who - according with our previous data - cluster with adverse hematologic characteristics, display here a higher capacity to over-express MCP-1 under an inflammatory stimulus and an impaired megakaryocytic differentiation potential. Further studies to better define the role of MCP-1 on CD34+-cells differentiation in the context of MF are desirable.

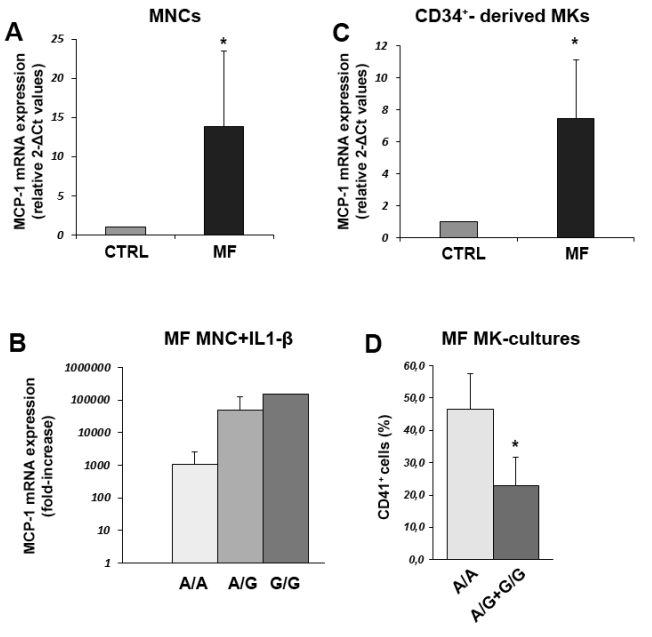

Figure legend
(A) MCP-1 mRNA expression in circulating MNCs from MF (n. 4) and CTRL (n.4) (data are expressed as relative $2^{-\Delta Ct}$ values as compared to CTRL).
(B) MCP-1 mRNA expression in MF-MNCs after ex-vivo IL1-β-stimulation according to patients' genotype (n. 3 A/A, n. 3 A/G and n. 1 G/G). Data are expresses ad fold-increase of $2^{-\Delta Ct}$ values from the baseline (T1 vs. T0).
(C) MCP-1 mRNA expression in MF (n. 5) and CTRL (n. 4) MKs obtained by ex-vivo differentiation of CD34+-cells (data are expressed as relative $2^{-\Delta Ct}$ values as compared to CTRL).
(D) Percentage of CD41+ cells obtained from megakaryocytic cultures of MF CD34+ cells at day 14, stratified according to MF patients' genotype (A/A, n. 3 vs. A/G+G/G, n. 3).

Figure 1.

CO064

IMMUNOMODULATORY EFFECTS OF IFNα ON T AND NK CELLS IN CHRONIC MYELOID LEUKEMIA PATIENTS IN DEEP MOLECULAR RESPONSE IDENTIFY POTENTIAL CANDIDATES FOR TREATMENT DISCONTINUATION

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Introduction: Deep and stable molecular response (DMR) is a pre-requisite for a successful treatment-free remission (TFR) in chronic myeloid leukemia (CML). Therefore, novel strategies aimed at activating the immune system to potentiate the activity of tyrosine kinase inhibitors (TKI) against residual leukemic cells may represent a promising approach. In order to better identify and analyze potential candidates of successful TFR, we have examined the phenotypic and functional host immune compartment in patients who had received TKI treatment only (TKI-only) or previously treated with INFα (IFNα+TKI).

Methods: We measured T/NK-cell subset distribution, NK and T-cell cytokine production, activation and maturation markers in CML patients in DMR eligible to TKI discontinuation according to recent NCCN and ESMO guidelines (stable MR4 for more than 2 years). We compared 10 patients treated with IFNα+TKI with 22 samples from TKI-only patients. Patients included in the study had discontinued IFNα for 15 years on average. T and NK cells have been evaluated for the surface expression of CD3, CD4, CD8, CD16, CD56, NKp30, NKp44, NKp46, NKG2C, NKG2D, DNAM-1, CD25, CD69, CD62L, CD57 and, after activation and permeabilization, for intracytoplasmic IFNγ and TNFα production (BD Biosciences, San Jose, CA and R&D System, Minneapolis, MN).

Results: We observed that IFNγ and TNFα were produced by a larger fraction of lymphocytes in INFα+TKI patients compared to TKI-only. In particular, the cytokine production by CD3+CD4+ cells was: 36.9 ± 16.8 vs 23.3 ± 9.4% for IFNγ (p=0.007), 70.4±11.0 vs 61.6 ± 20.2% for TNFα (p=ns) and by CD3+CD8+: 75.2 ±17.4 vs 64.2 ± 18.2% for IFNγ (p=ns), 71.3±17.1 vs 62.2 ± 21.1% for TNFα (p=ns). No differences were revealed in the percentage or ratio of CD4+ and CD8+ T cells among the two groups or in the percentage of NK and NK-T cells, nor in the NK cell subpopulations (CD56bright/CD16-, CD56bright/$CD16^{dim}$, $CD56^{dim}$/CD16+), as well in the activation, maturation markers and activating receptors (NKp30, NKp44, NKp46 and NKG2D) for NK cell subsets. In INFα+TKI patients, the NKG2C mean fluorescence intensity (MFI) was significantly higher compared to the TKI-only group in the $CD56^{dim}/CD16^{+}$ NK cell subset (p=0.03). Furthermore, we observed a significant increase of DNAM-1 MFI in the $CD56^{bright}/CD16^{-}$ NK cell subset (p=0.02).

Conclusions: Our data indicate that previous exposure to IFNα substantially and persistently modified the immune system of CML patients in memory T lymphocytes, differentiated NKG2C+ "long-lived" NK cells and DNAM-1+ "adaptive" NK cell responses, even after a long time period (more than 15 years) from the last IFNα contact. Our results confirm that IFNα modulates and potentiates the host immunologic compartment and pave the way to design and carry out immunotherapeutic strategies aimed at achieving and maintaining a DMR before and after TKI discontinuation in CML patients.

Monoclonal Gammopathies and Multiple Myeloma 2

CO065

ANALYSIS OF MUTATIONS AND STRUCTURAL VARIANTS TO REDEFINE THE GENOMIC LANDSCAPE OF MULTIPLE MYELOMA AND ITS CLINICAL IMPLICATIONS

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Introduction: In multiple myeloma (MM), karyotypic events such as translocations between the IGH locus and known oncogenes, and recurrent copy-number abnormalities (CNAs) are considered early drivers, being detectable also in pre-malignant stages of the disease. Recently, several recurrent single-nucleotide-variants (SNVs) have been described in MM, but their real driver role and relationship with other genomic events have never been explored on large series.

Methods: Here, we combined whole genome (n=30), whole exome (n=849) and targeted (n=373) sequencing data of 1252 MM patients. Eight hundred and four patients were included from the CoMMpass study, generated as part of the Multiple Myeloma Research Foundation Personalized Medicine Initiatives. The driver vs passenger role of each SNV was defined by the dNdS algorithm (Martincorena *et al.*, Cell 2017). The hierarchical dirichlet (HDP) process was used to investigate the main MM genomic subgroups as previously described (Bolli *et al.* Leukemia 2017).

Figure 1.

Results: Combining WGS and 879 whole-exome data, we extracted 56 significant driver SNVs [median of 1 per patient (range 0-6)], with KRAS (23%), NRAS (22.1%), DIS3 (9.5%) and FAM46c (4.8%) confirmed as the most recurrent. At least one driver SNV was extracted in 741 patients (84%). We then included additional 373 MM patients investigated by an unmatched targeted sequencing approach (Bolli *et al.* Leukemia 2017), to create the largest dataset of MM samples to date (n=1252) to investigate the interrelationships of karyotypic events (n=14) and the most frequent SNVs (n=21). To this end, patterns of co-occurrence and mutual exclusivity of recurrent CNAs and SNVs were derived from their distribution and clustered using the HDP. Karyotypic events contributed to clustering more than SNVs, and we extracted five main clusters based on their extended genotype (Figure 1). The first was defined by hyperdiploidy and accounted for 59% of the entire series. del13q, del TRAF3, gain1q21 and del1p13 defined the second cluster (18%). t(11;14)(CCND1;IGH) and mutated NRAS/KRAS defined the third cluster (11%). del13q, gain 1q21, DIS3 mutation, t(4;14) defined the fourth cluster (5.5%). TP53 mutation, del17p13, del13q14, t(11;14), deletion of CYLD defined the last cluster (4%). With a median follow-up of 621 (range 31-4205) days, the clusters had a distinct clinical outcome, with cluster 5 showing the poorest overall survival and cluster 3 showing a favorable outcome.

Conclusion: Our data show that a tentative genomic classification in MM is dominated by karyotypic events, with driver SNVs occurring during later on distinct genomic profiles. Our analysis showed significant clustering, however most events were not entirely segregated within each group, suggesting a context-dependent effect of many of them, and a role for other genomic non-coding drivers. Our analysis supports the use of extended genotyping of MM cases at diagnosis for classification and prognostication.

CO066

THE EXOSOMES-DERIVED EGFR LIGAND AMPHIREGULIN (AREG) IS A NEW KEY PLAYER IN MULTIPLE MYELOMA BONE DESTRUCTION

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Introduction: Multiple Myeloma (MM) cell-derived exosomes (exo) play a relevant functional role in the induction of osteoclast (OC) differentiation and activity. Recent data indicate that the epidermal growth factor receptor (EGFR) system plays important roles in bone remodeling and that EGF-like ligands stimulate osteoclastogenesis by acting at least in part on osteoblasts (OBs). The aim of this study is to identify EGFR ligands in MM cell exo and to determine if they are able to functional modulate the tumor microenvironment by affecting OC and OB functions.

Methods: Exo were isolated from the conditioned medium (CM) of human myeloma cell lines (HMCLs), MM1.s and RPMI-8226, and from bone marrow (BM) plasma of 4 MM patients (3 newly diagnosed and 1 relapsed; 50% with bone disease). Exo were characterized for their dimensions and their contents identified by western blotting, focusing on the EGFR ligand Amphiregulin (AREG). To test MM-exo effect on osteoclastogenesis, primary CD14+ monocytes purified from healthy donors peripheral blood and the murine cell line RAW264.7 were used as OC models. Moreover, human TERT-transfected mesenchymal stromal cells (hTERT-MSCs) were used to evaluate the role of MM exo on OB differentiation. Cells were treated with (i) MM exo (25 ug/ml) in the presence or absence of anti-AREG neutralizing antibody (nAb) (20 ug/ml) or (ii) with recombinant human (rh) AREG (20 ug/ml). OC and OB differentiation markers were measured by Real-Time PCR and ELISA assay. Moreover, hTERT-MSCs were co-cultured with MM cells in the presence or absence of anti-AREG nAb for 48 hours and the CM collected for osteoprotegerin ELISA assay. A lentiviral vector was used to inhibit in monocytes the expression of SNAI1, target gene of AREG signaling pathway, and test the effect on OC differentiation.

Results: Physical characterized of MM-derived confirmed that we are working with exo of about 80 nm. AREG was specifically enriched in exo derived from both MM cells and BM plasma of 3 MM patients. We found that exo-derived AREG led to the activation of EGFR in pre-OC, as showed by increased SNAI1 mRNA expression in both RAW264.7 (exo vs control $p=0.043$) and CD14+ cells (exo vs control $p\leq0.05$); the presence of anti-AREG nAb reverted this effect (exo nAb vs exo $p\leq0.05$). In addition, the treatment with MM-derived exo significantly increased the expression of OC specific markers compared to controls, and this effect was reverted by anti-AREG nAb (exo nAb vs

exo p≤0.05). Lastly, we found that the treatment of hTERT-MSCs with MM-derived exo reduced the expression of OB markers, leading to the inhibition of cell differentiation.

Conclusion: Our data indicate that AREG, packed into MM-derived exo, is involved in both OC formation and inhibition of OB differentiation and it could be responsible for the uncoupled bone remodeling induced by MM cells. In conclusion, AREG represents a potential new player in MM-induced bone destruction.

CO067

CHARACTERIZATION OF RP11-760H22, A NOVEL LNCRNA DEREGULATED IN MULTIPLE MYELOMA

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Introduction: MM is a malignant proliferation of bone marrow PCs characterized by highly heterogeneous genetic instability. During the last decades lncRNAs relevance in cancer has been highlighted by a large number of studies. In previous reports, we demonstrated that deregulated patterns of lncRNAs expression are associated with distinct MM molecular subtypes (Ronchetti *et al.*, Oncotarget, 2016; Ronchetti *et al.*, Sci Rep 2018). Here, we report the preliminary characterization of the lncRNA RP11-760H22, a novel transcript located at the 3' end of DEPTOR gene, known as an important player involved in myelomagenesis for sustaining cell proliferation and survival and for the maintenance of the cellular ER function.

Methods: lncRNA transcriptional profiles were generated on GeneChip® Human Gene 2.0 ST microarray in purified bone marrow PCs (> 90%) from 50 MM, 15 PCL and 4 normal donors. qRT-PCR has been used to validate array data. lncRNAs subcellular localization was evaluated by fractionated HMCLs nuclear and cytoplasmic RNA. HMCLs were treated with actinomycin D to determine the half-life of the lncRNAs. HMCLs silencing by the use of GapmeR and siRNA of RP11-760H22 and DEPTOR gene respectively was performed by Neon Transfection system; the efficiency of silencing was evaluated by qRT-PCR. Luminometric assay was used to investigate cell viability and caspase cleavage upon silencing. Modulation of DEPTOR protein expression levels was evaluated by WB technique.

Results: RP11-760H22 maps to chr. 8 and is located in sense at the 3' end of DEPTOR gene which encodes a protein markedly overexpressed in MM and conferring a strong proliferative and survival advantage to malignant PCs. The microarray transcriptional analysis showed that both DEPTOR mRNA and RP11-760H22 are significantly upregulated in pathological samples compared to healthy controls and are strongly correlated at expression levels. In particular, RP11-760H22 and DEPTOR are highly overexpressed in a subset of multiple myelomas harboring cyclin D1/D3 or c-MAF/MAFB translocations. Analysis on HMCLs revealed that RP11-760H22 is equally distributed in the nuclear and cytoplasmic cellular fractions, showing a long half-life similarly to DEPTOR mRNA. siRNA silencing of DEPTOR gene in HMCLs led to a significant down-regulation of RP11-760H22 and it is associated with caspases 3/7 cleavage and increase of mortality. Furthermore, preliminary experiment related to RP11-760H22 silencing in HMCLs using in-house design LNA-gapmeR led to a downregulation of both lncRNA and mRNA thus suggesting a possible co-regulation mechanism.

Conclusions: Our data indicate that RP11-760H22 is significantly upregulated in MM patients and that is highly correlated with the expression of DEPTOR gene. Our preliminary results suggest a possible co-regulation mechanism between the lncRNA and DEPTOR prompting us to investigate the role of lncRNA RP11-760H22 in one of the most important molecular pathway involved in myelomagenesis.

CO068

HIGHER LEVELS OF GENOMIC COMPLEXITY CORRELATES WITH AN ADVANCED PLASMA CELL DIFFERENTIATION STATUS IN NEWLY DIAGNOSED MULTIPLE MYELOMA PATIENTS

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Introduction: Plasticity is a hallmark of Multiple Myeloma (MM) clone(s), where both quiescent MM cells, acting as tumor-initiating cells, and proliferative MM cells, able to invade and disseminate, might coexist. How cells switch from one state to the other and the diverse cell states balance remains unclear. Aim of the study is to correlate the genomic background with phenotypic plasticity of MM clone(s) at diagnosis, in order to stratify patients (pts) according to both the level of chromosomal instability (CIN) and their plasma cells (PCs) differentiation stages, and to evaluate the impact of this stratification on the disease outcome.

Patients and Methods: 145 newly diagnosed MM pts were included in the study. Whole-genome copy number alterations (CNAs) were analysed by SNP array both in the CD138+PCs and CD19+B-cells. In each pts, both the CD138+/CD38high PCs and CD19+B-cells compartments were characterized by 6-color multi-parameter flow cytometry analysis, combining CD138-PE, CD38-PE-Cy7, CD20-APC, CD19-APC-Cy7, CD27-FITC, CD45-FITC, CD28-APC, CD44-FITC, CD54-APC, CD81-PerCP-Cy5.5, CD56-APC.

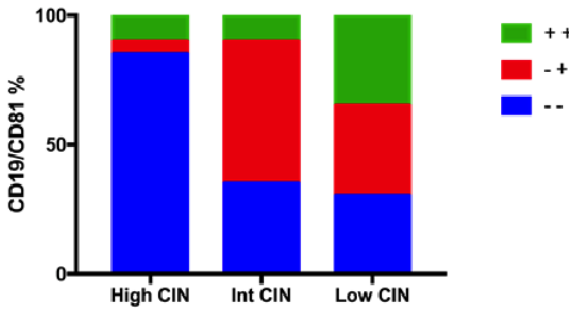

Fig.1 Stratification of MM patients according to different levels of chromosomal instability (CIN). For each subgroup the differentiation stages distribution of the is indicated: CD19-/CD81- (blue), CD19-/CD81+ (red) and CD19+/CD81+ (green).

Figure 1.

Results: According to the CD138+ PCs' CIN, as described both by the total amount of CNAs and by the portion of genome changed (GC), three major pts subgroups were identified (Fig.1): the most representative (33%), was characterized by a higher CIN (median CNAs: 550 %GC ≥ 25) as compared to the others (intermediate and low CIN, median CNAs: 220 10≤ GC%≥25). Hyperdiploidy, but also high-risk features [i.e. 1p del (FAF1), 16q del (WWOX, FANCA) and 17p del (TP53) mainly characterized CD138+ PCs with high CIN. On the contrary, in the same pts, the CD19+B-cells display a quite simple karyotype with very few microalterations (>50kb), mostly involved in the signal transduction pathway (loss on KRAS, chr12p12.1 and on SIRPB1, chr20p13). According to the the co-expression of CD19/CD81, describing the MM clone(s) differentiation status, PCs with a high level of CIN resulted more mature CD19-/CD81-. Both the high expression of CD28 and CD44 and the reduced expression of CD20, CD27 and CD45 confirmed the advanced differentiation status, as well as the decrease of CD138-/low/CD19+/CD20-/CD38high/CD27high plasmablast population, sug-

gesting a reduced, more quiescent, B cells reservoir pool. Finally, although baseline clinical features of pts with more mature, genomically instable PCs, are associated to bad prognosis (e.g. PET lesions, k/l ratio, ISS III, β2-microglobulin; p<.05), they are more likely to obtain high quality response rates (≥CR) to PI induction therapy.

Conclusions: High level of genomic complexity correlates with advanced PCs differentiation stages, and this is lastly associated with a prevalence of poor prognosis features. Both CIN and phenotypic pliancy represent important, yet poorly defined, mechanisms by which MM clone(s) accelerate their own evolution and survival.

Acknowledgements: AIRC, AIL, Fond. Berlucchi.

CO069

ARGININE SHORTAGE DUE TO EXPANSION OF PMN-MDSC IS DISPENSABLE FOR BORTEZOMIB RESISTANCE IN MULTIPLE MYELOMA

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Background: Despite improvement in overall response due to the introduction of the first-in-class proteasome inhibitor bortezomib (btz), multiple myeloma (MM) is still an incurable disease due to the immune-suppressive bone marrow (BM) environment. Our previous work showed that MM environment can educate myeloid precursors to become immune-suppressive and that myeloid-derived suppressor cells are increased upon disease progression from MGUS through MM. We aimed to identify the contribution of CD11b+CD15+CD14-HLA-DR- granulocytic-like myeloid-derived suppressor cells (PMN-MDSC) in MM patients treated up-front with novel agents bortezomib and lenalidomide. Then, in three MM cell lines (MM1.s, U266 and OPM2) and primary cells derived by MM we investigated sensitivity to bortezomib and lenalidomide in presence of PMN-MDSC and its main bio-biomarker Arginase-1 (Arg-1).

Results: We found that PMN-MDSC and their function through increased arginase-1 (Arg-1) and reduced arginine are associated with MM progression. Indeed, Arg1+PMN-MDSC were increased in peripheral blood of newly diagnosed MM patients compared to healthy subjects. Flow cytometry evaluation of both peripheral blood and bone marrow disclosed that the main source of Arg-1 were PMN-MDSC, while mo-MDSC, T-cells or CD138+ plasma cells virtually did not express Arg-1. When we assessed cell viability of the human myeloma cell lines MM1.s, OPM2 and U266 treated with 5-20 nM btz for 24 h in PMN-MDSC conditioned media, we disclosed that amount of Arg-1 and Arg-1 inhibition could affect btz sensitivity in-vitro, in a dose-dependent synergic way. PMN-MDSC and Arg-1 were reduced after exposure to lenalidomide-based regimen but increased after btz-based treatment, suggesting that btz sensitivity could be further increased by associating Arg-1 inhibition. Progressive arginine deprivation (1 μM-10 nM) *in vitro* induced an adaptive response through the engagement of GCN2 signalling and autophagy induction within 24 hours. At lower arginine concentrations, MM cells could still proliferate *in vitro*, even in low-glucose media, while T-cells do not. Arginine deprivation or treatment with human recombinant arginase-1 (which reduced extra-cellular arginine availability within 12 hours of exposure) alleviated the cellular glucose dependence by increasing glutamine anaplerosis for mitochondrial ATP generation, without affecting btz sensitivity.

Conclusions: In MM, Arg-1 is mainly expressed by PMN-MDSC. PMN-MDSC and Arg-1 are reduced *in vivo* after lenalidomide but not bortezomib treatment. The consequent arginine deprivation induces an adaptive response in MM cells alleviating the cellular glucose dependence by increasing glutamine anaplerosis for mitochondrial ATP generation, without affecting btz sensitivity.

CO070

MYC-MIR-22 REGULATORY LOOP MODULATES LENALIDOMIDE ACTIVITY IN MULTIPLE MYELOMA CELLS

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Introduction: In our previous work, we showed that miR-22 acts as tumor suppressor-miRNA in Multiple Myeloma (MM) via targeting a novel DNA repair addiction of neoplastic plasmacells. Here, we provide novel insights into transcriptional mechanisms suppressing miR-22 expression in MM.

Methods: Cell proliferation and apoptosis were evaluated with CellTiter-Glo assay and Annexin V staining. c-MYC, PARP1, Caspase-3 levels were analyzed by Western blot of whole protein extracts, using GAPDH as loading control. miR-22 levels were evaluated by qRT-PCR after normalization on RNU44. For chromatin immunoprecipitation (ChIP) experiments, the ChIP Assay Kit (Pierce Agarose ChIP Thermo Fisher Scientific) was used. Gene expression profiles were obtained from AMO-1 cells 24h from transfection with miR-22 or miR-NC. Microarray data was generated by GeneChip® Human Transcriptome 2.0 Array (Affymetrix Inc., Santa Clara, Ca).

Results: In silico search of transcription factors (TF) binding sites within miR-22 promoter revealed a putative MYC consensus. By interrogating public available MM patient dataset, we found a significant inverse correlation between MYC and miR-22 expression. Consistently, MYC inhibition mediated by specific siRNA or validated MYC inhibitor such as JQ1 or 10058-F4, induced significant increase of miR-22 levels. Indeed, using ChIP assay, we confirmed significant enrichment of MYC at miR-22 promoter in MM cells, thus confirming that c-MYC could repress miR-22 expression by inhibiting its transcription. Then, by performing Gene Expression Profiling (GEP) and Gene Set Enrichment Analysis (GSEA) after miR-22 overexpression, we found and validated a significant reduction of MYC activity, partly mediated by miR-22 targeting of MYCBP, a positive regulator of c-MYC, thus establish a new feedback loop in MM. Lenalidomide, immunomodulatory and anti-proliferative agent with significant activity in a range of haematological disorders, including MM, decreases MYC transcription resulting in growth inhibition of myeloma cells. Consistently with this mechanism of action, we found that Lenalidomide increased miR-22 expression in a MYC dependent manner. Finally, we demonstrated that miR-22 increased Lenalidomide-blockade of MYC signaling, resulting in a synergistic cytotoxic effect in Myc-driven haematological malignancies, including Bortezomib-resistant MM and Diffuse Large B Cell Lymphoma (DLBCL) cells.

Conclusions: Taken together, our findings indicate that: a) Down-regulation of miR-22 in MM is in partly due to MYC repression of miRNA transcription; b) miR-22 reduces oncogenic transcriptional activity of c-MYC, by targeting MYCBP; c) anti-MM effects of Lenalidomide is partly mediated by miR-22 de-repression, which triggers a novel Myc-dependent synthetic lethality.

CO071

IN MULTIPLE MYELOMA CIRCULATING PLASMA CELLS ARE EXPRESSION OF MORE AGGRESSIVE DISEASE AND THEIR IDENTIFICATION BY FLOW CYTOMETRIC SINGLE PLATFORM IS RELATED TO NEGATIVE PROGNOSTIC PARAMETERS

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Introduction: Recent studies showed the prognostic role of peripheral circulating plasma cells (CPC) both in newly diagnosed multiple myeloma (MM) and in smoldering MM but to date CPC are detected by low reproducible and sensitive methods. CPC are considered a marker of more severe disease and are characterized by the expression of surface molecules such as CD184 (CXCR4) that, involved in PC homing, enhances the acquisition of aggressive phenotype. For the first time we performed a single platform absolute CPC count. Afterwards, we compared them with patients' baseline characteristics and valued their CD184 expression.

Method: 413 peripheral samples were collected by newly diagnosed MM patients enrolled in the UNITO-MM-01/FORTE. For the single platform tube the following antibodies CD38PC7/CD138PC5.5/ CD45KO/ CD184APC/CD56PE/CD19PB were mixed with 100 uL of EDTA peripheral blood dispensed with reverse pipetting (RP), added with 500 uL of lysing solution and, after 15 min, 100 uL of flow count were dispensed with RP and acquired by Navios flow cytometer. To reduce the acquisition of cellular debris, a "live gate" was set up in order to exclude events CD38 and CD45 negative. The CPC clonality was confirmed, in a second tube, by the intracytoplasmic staining of kappa and lambda light chains.

Results: CPC were detected in 94.4% of patients with median values of 0.03% (range 0%-51%) and 2.37/mm^3 (range 0/mm3-6272/mm^3). CPC absolute values were sorted in quartiles (0/mm3-0.86/mm^3, 0.86/mm^3-2.37/mm^3, 2.37/mm^3-11.2/mm^3, 11.2/mm^3-6272/mm^3) and associated with poor prognostic features. Significant association, expressed by Cramer's V >0.2, were observed between CPC and: hemoglobin (V= 0.41), ISS (V= 0.26), R-ISS (V= 0.24), ≥60% of PC in biopsy (V= 0.23), bone marrow aspirate (BM) PC sorted in quartiles (V= 0.21), LDH upper the upper limit (V= 0.24) all with a p-value <0.001. By surface and intracytoplasmic antigen expression patients were classified into three groups: 200 patients with monoclonal CPC (M CPC) only, 133 with policlonal CPC (P CPC) only, 73 with both CPC (MP CPC). The medians of CD184 Mean Fluorescence Intensity (MFI) in bone marrow PC did not show statistical significant differences among these three groups (M: 11.6, P: 10.7, MP: 8.2 p=0.08) and did not correlate to CPC number. CD184 median MFI of M CPC (49.98) was higher than P CPC (33.6) (p<0.01) and CD184 MFI of BM PC was lower than both CPC categories (10.8) p<0.001.

Conclusions: The single platform method easily and quickly quantified CPC in 94.4% of PB MM samples and the absolute numbers did well associate with negative prognostic factors confirming their role as a marker of highly aggressive disease. Monoclonal CPC showed a CD184 higher expression than BM PC and this result confirms, as stated by other studies, that in this population an epithelial-mesenchimal-like transition occurs that confers more aggressive features in terms of disease dissemination.

CO072

THE LOSS OF CD38 EXPRESSION BY MYELOMA PLASMA CELLS MAY OCCUR IN THE EXTRAMEDULLARY DISEASE

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Introduction: Extramedullary disease (EMD) is defined by the presence of clonal plasma cells (PCs) in a site outside of the bone marrow (BM) in a patient with multiple myeloma (MM). A variable expression of adhesion molecules, including CD44 and CD56, has been hypothesized in the pathophysiology of the extramedullary spread. CD38 is a transmembrane glycoprotein, highly and uniformly expressed by BM PCs, which plays a dual role as adhesion molecule and ectoenzyme. Recently, anti-CD38 targeted monoclonal antibodies, such as Daratumumab, have been included in the therapeutic armamentarium of MM. CD38 is considered a hallmark of MM cells however its expression by extramedullary PCs is still unknown.

Methods: In this study to define the CD38 expression profile in the EMD we investigated 17 patients (median age at diagnosis 68 years, range 47 – 76), treated at our Institution from 1999 to 2018. All patients were affected by plasma cell dyscrasia (15 MM and 2 primary plasma cell leukemia, PCL) and presented a biopsy proven EMD. An analysis of clinical, immunohistochemical and immunophenotypic features, focusing on CD38 expression, was performed.

Results: Among the 17 patients evaluated, 14 patients presented EMD at relapse and 9 patients showed an involvement of more than one organ site. The most common sites of EMD were soft tissues (26%), liver (18%), lymph-nodes (18%) and testis (9%). The most frequent chromosomal abnormality observed was 13q deletion in about 60% of patients. High risk FISH abnormalities (del17p, t (4;14), t (14;16), t (14;20)) were reported in 20% of patients. The median time from initial diagnosis to extramedullary relapse was 29 months (range 9-208 months). In 10 patients EMD spread appeared during treatment with new drugs based regimens, which included first and second generation proteasome inhibitors and immunomodulatory drugs. Nobody was under treatment with Daratumumab at the moment of EMD. The expression of CD38, CD56 and CD44 was evaluated by immunohistochemistry in the EMD and BM samples. Interestingly, we found that 5 out of 17 patients, the 29% of our entire cohort, showed a markedly reduced or a lack of expression of CD38 at the extramedullary sites. At diagnosis, in these cases, CD38 was positive on BM PCs detected by flow cytometry or immunohistochemistry. In one relapsed patient, with both data available, the lack of CD38 expression in EMD sample was demonstrable only by immunohistochemistry, with a weak CD38 surface expression detected by flow cytometry. He was treated with one cycle of Daratumumab and showed a clinical progression.

Conclusions: Our data indicates that the loss of CD38 expression may occur in EMD with a possible impact on the therapeutic options. In the era of anti-CD38 immunotherapy a tissue biopsy should be undertaken to document CD38 positivity in EMD sites of MM patients.

Acute Leukemia 2

CO073

CRLF2 OVEREXPRESSION: NEW "TRIPLE NEGATIVE" GENE EXPRESSION CLUSTERING TO IDENTIFY NEW ADULT ACUTE LYMPHOBLASTIC LEUKEMIA SUBGROUPS

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Background: The heterogeneous and poor survival group of Philadelphia negative (Ph-) B-ALL patients (pts) that doesn't have the most recurrent adult rearrangements (BCR-ABL1 t(9;22); TCF3-PBX1 t(1;19); MLL-AF4 t(4;11)) are collectively referred to as "triple negative" (Ph-/-/-) ALL. CRLF2 is frequently altered in adult B-ALL, especially in Ph-like pts (50-75% of cases). Alterations that lead, in the majority of cases, to a CRLF2 overexpression. Adult pts with CRLF2 upregulated have poor outcome and novel strategies are needed to improve it. Aims: Clustering and biological characterization of Ph-/-/- ALL, considering CRLF2 overexpression event, in order to define and assess biomarkers in this subgroup to test new drugs. Patients and Methods: Gene Expression Profiling (GEP; HTA 2.0 Affymetrix) were performed on 55 Ph-/-/- ALL, 29 B-ALL Ph+ at different time point of the disease and on 7 mononuclear cell of healthy donors. Data were normalized with the Expression Console Software. Successively we cluster triple negative GEP data with our validated pipeline, based on CRLF2 upregulation and in the top ten-gene list. Ph-/-/- ALL samples were then characterized for the presence of gene fusions, Copy Number Alterations (CNAs) and mutations using different approaches (TruSight Pancancer-Illumina; MLPA and/or dMLPA-MRC-Holland; SNP Array-Affymetrix and PCR). Results: Clustering our Ph-/-/- gene expression data using the impact of the 10 single genes in our cohort, we could identify a defined 2-clusters-subdivision (Gr1 and Gr2). The Gr2 is characterized by CTGF, CRLF2 and CD200 (Gr2=3C-up) overexpression and it represents 14.1% of all B-ALL. The Gr2 GEP is similar to Ph+ one. Fusion and mutational screening done, detected that Gr2 has a higher frequency of Ph-like associated lesions, that mainly affect JAK-STAT pathway. Also IKZF1 and EBF1 deletions are significantly associated to Gr2 (p=0.003; p=0.016). RAS pathway genes are highly affected in Gr1. Notably p53 pathway is enriched in both groups but with different deregulated genes: CHEK2 is upregulated in the group1 and CDK6 in the gr2. Conclusions: We identified a new signature, related to CRLF2 high expression, to classify Ph-/-/- ALL B-based on 10 genes. 3C-up represents 14.1% of all B-ALL and it is characterized by a) high co-expression of three main genes: CRLF2, CTGF and CD200; b) IKZF1 deletion; c) JAK-STAT pathway mutations/fusions/deletions. Gr1 represents 46.9% of all B-ALL. Gr2 GEP similarity to Ph+ one, suggests that this Gr2 could contain Ph-like pts. This new Ph-/-/- subclassification identify new potential therapeutic targets with available drug (α-CTGF, α-CD200, CDK2, CHK2 and CDK6 inhibitors; tyrosine kinase inhibitors already effective on Ph+ and Ph-like) to test. Supported by: ELN, AIL, AIRC, project Regione-Università 2010-12 (L. Bolondi), FP7 NGS-PTL project, HARMONY project, Fondazione del Monte BO e RA project.

CO074

SENSITIVE AND EARLY DETECTION OF THE PML-A216V MUTATION BY DROPLET DIGITAL PCR IN ARSENIC TRIOXIDE RESISTANT ACUTE PROMYELOCYTIC LEUKEMIA

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Introduction: The use of ATRA combined with chemotherapy (CHT) or arsenic trioxide (ATO) can induce long-term remissions in the vast majority of patients with newly diagnosed APL. Despite this therapeutic success, 5-10% patients still relapse. Up to 30% of relapsed/refractory patients have been shown to harbor point mutations within the ATO-binding domain (B2), in particular at position A216 of the PML moiety of the PML/RARA hybrid. These alterations affect the binding of ATO to PML and thus impair PML/RARA degradation and clinical response to this agent. We developed a droplet digital PCR (ddPCR) assay for the sensitive detection of PML-A216V mutation in relapsed APL patients as a tool to early predict ATO-resistance.

Methods: A total of 13 patients who relapsed after ATO treatment were analyzed (Table 1). Initial treatment included ATRA/CHT in 10 and ATO/ATRA in 3 cases. Eleven patients in the series presented multiple relapses. Mutational analysis was performed at the time of first relapse in the 3 patients treated with front-line ATO and in ≥2nd relapse in patients receiving ATO as salvage treatment. The limit of detection (LOD) of ddPCR assay was determined by diluting mutant DNA in the DNA sample derived from a healthy donor. Samples were considered positive if they had ≥3 positive droplets above the threshold of the negative template controls. The ddPCR assay was initially performed on DNA samples collected at relapse, in presence of high PML/RARA copy number by conventional RQ-PCR. We then investigated the mutation dynamics by backtracking the identified mutation in samples collected for routine PML/RARA monitoring prior to relapse. To confirm and compare the results obtained by ddPCR, all positive samples were also analyzed by Sanger sequencing.

Table 1. Clinical and biological characteristics of APL patients at diagnosis.

UPN	Age/Sex	Sanz risk	PML/RARA isoform	First-line treatment	CR1 duration	Relapses (n)	Timing of ATO treatment	Response to ATO	*PML* mutational status by ddPCR
1	54/M	Standard	BCR1	ATRA-ATO	3 months	2	Front-line	mCR*	*PML* wt
2	32/M	Intermediate	BCR1	ATRA-ATO	23 months	1	Front-line	mCR*	*PML* wt
3	77/M	Intermediate	BCR3	ATRA-ATO	14 months	1	Front-line	mCR*	*PML* wt
4	29/F	Intermediate	BCR1	ATRA-CHT	26 months	4	Salvage	Refractory	***PML* A216V**
5	77/M	Intermediate	BCR1	ATRA-CHT	17 months	2	Salvage	Refractory	***PML* A216V**
6	40/F	Intermediate	BCR2	ATRA-CHT	3 months	1	Salvage	Refractory	***PML* A216V**
7	80/M	Intermediate	BCR1	ATRA-CHT	24 months	2	Salvage	Refractory	***PML* A216V**
8	30/M	Standard	BCR3	ATRA-CHT	8 months	2	Salvage	Refractory	*PML* wt
9	42/F	High	BCR3	ATRA-CHT	9 months	3	Salvage	Refractory	*PML* wt
10	42/F	Low	BCR3	ATRA-CHT	12 months	2	Salvage	mCR*	***PML* A216V**
11	61/M	Intermediate	BCR3	ATRA-CHT	30 months	3	Salvage	Refractory	*PML* wt
12	66/F	Intermediate	BCR1	ATRA-CHT	26 months	3	Salvage	Refractory	*PML* wt
13	32/F	Standard	BCR1	ATRA-CHT	36 months	3	Salvage	mCR*	*PML* wt

*mCR= molecular Complete Remission

Results: The ddPCR test showed high reproducibility and sensitivity and was able to detect down to 0.4% PML-A216V-mutant allele fraction. After assessing the false positive rate (FPR), the A216V mutation was detected by ddPCR in 5/13 patients (38%) who relapsed after ATO. The ddPCR assay carried out in follow-up DNAs of mutated patients (total of 44 samples, median 3 per patient, range:1-22), revealed the presence of PML-A216V mutation in 17 samples. Of these, 3 were collected in overt relapse while 14 were taken earlier, at molecular relapse in patients with low PML/RARA transcript levels. Sanger sequencing confirmed the mutation in 4/17 samples only. In 3 mutated patients for whom several sequential samples were available, a positive-ddPCR test anticipated a positive

Sanger sequencing result by 3, 4 and 24 months, respectively.

Conclusions: Our data show that a ddPCR assay can be efficiently employed in the screening of PML-A216V mutation in APL and is able to identify mutant cases earlier in the disease course as compared to conventional sequencing, helping to identify ATO-resistant APL patients who are candidates to alternative treatment strategies.

CO075

THE IDENTIFICATION OF G9A/EHMT2 REVEALS A NOVEL INTERACTION BETWEEN EPIGENETICS AND METABOLISM IN T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA

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Introduction: The identification of leukemia epigenetic mechanisms has fueled the search of drugs that target enzymes involved in DNA methylation and post-translational histone modification. Although, the epigenetic-metabolomics interplay that sustains cancer cell proliferation, pluripotency and resistance to therapy are far to be dissected. Here we characterize G9a/EHMT2, a protein lysine methyltransferases, as an epigenetic and metabolic target in T-cell acute lymphoblastic leukemia (T-ALL).

Methods: To identify new targets in T-ALL we intersected an epigenome-centered shRNA and a low throughput small molecules screen of epigenetic modifiers with the computational analysis of EHMT2 expression in publically available cancer and normal tissue databases. G9a/EHMT2 emerged as "druggable" target in T-ALL. To demonstrate that G9a is required for T-ALL growth we performed both chemical and genetic studies and described the phenotypic consequences of G9a loss. Morphological appearance of cells treated with G9a inhibitors guided functional and biochemical experiments to justify the role of G9a in the regulation of glycogen metabolism.

Results: We previously demonstrated that G9a/GLP inhibitors significantly inhibited cell viability compared to other epigenetic modifiers including histone acetyltransferase (HAT), p300 modulators, EZH2 and DOT1L inhibitors. G9a/EHMT2 and GLP/EHMT1 are conserved protein lysine methyltransferases that localize in euchromatin regions and regulates gene expression and chromosome structure through de novo mono- and dimethylation of histone H3 lysine 9. Furthermore, the intersection of publicly available databases demonstrated that EHMT2 is a preferential marker of lymphoid differentiation and that is highly expressed in T-ALL compared to other cancer subtypes or healthy bone marrow cells. Moreover, an epigenome-centered shRNA screen and low-throughput sgRNA CRISPR validation studies confirmed that T-ALL proliferation depends on G9a expression. We showed that inhibition of G9a impairs T-ALL viability and triggers the formation of cytoplasmic vacuoles, lysosomes and apoptotic bodies as demonstrated by transmission electron microscopy (TEM). These autophagic vacuoles resulted positive to the Periodic Acid-Schiff reaction and together with the detection of electrodense bodies observed in TEM strongly supported the evidence of intracellular glycogen granules accumulation. Consequently, we speculated that G9a loss might alter glycogen metabolism. Thus, we measured the activity of glycogen synthase kinase-3 (GSK3) by western blotting. We observed G9a inhibition causes an increase of the phospho-Ser9/21 GSK3 (inhibitory sites) upon drug treatment, hence promoting glycogen synthesis.

Conclusions: The intersection of multiple chemical-genetic-computational approaches led to the identification of G9a/EHMT2 as a target in T-ALL and revealed a new epigenetic control of glycogen metabolism in this aggressive subset of leukemia.

CO076

IMMUNOPHENOTYPIC PROFILING OF LEUKEMIC STEM CELLS TO TRACK FLT3-ITD POSITIVE, CHEMO-RESISTANT CLONES IN ACUTE MYELOID LEUKEMIA

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Introduction: Persistence of leukemia stem cells (LSCs) in AML patients achieving complete remission after chemotherapy lead to disease recurrence and poor outcome. Therefore, the identification of LSCs driving resistance to therapy represents an important challenge. LSCs reside within the CD34+/CD38- cells and several groups have identified cell surface antigens preferentially expressed on this population. We demonstrated a strong correlation between the CD123/CD99/CD25+ population within CD34+ cells and the presence of the FLT3-ITD mutation, associated with higher risk of AML relapse.

Aim: The aim of this study was to characterize the LSCs of FLT3-ITD positive patients to trackthe expansion of mutated clones.

Methods: Bone marrow (BM) samples from 9 patients with de novo FLT3-ITD positive AML were analysed by 8-color flow cytometry. A sequential gating strategy was carried out to purify the hematopoietic precursor fraction CD34/CD45/CD123+,CD38-, the LSCs enriched fraction CD34/CD123/CD99/CD25+, the CD34+ stem cell subset (CD123/CD99/CD25-) and T-lymphocytes. BM cells from UPN9 were sorted at diagnosis, during treatment and at the relapse. Cells were purified by a high-speed cell sorter. FLT3-ITD monitoring was carried out through PCR followed by capillary electrophoresis.

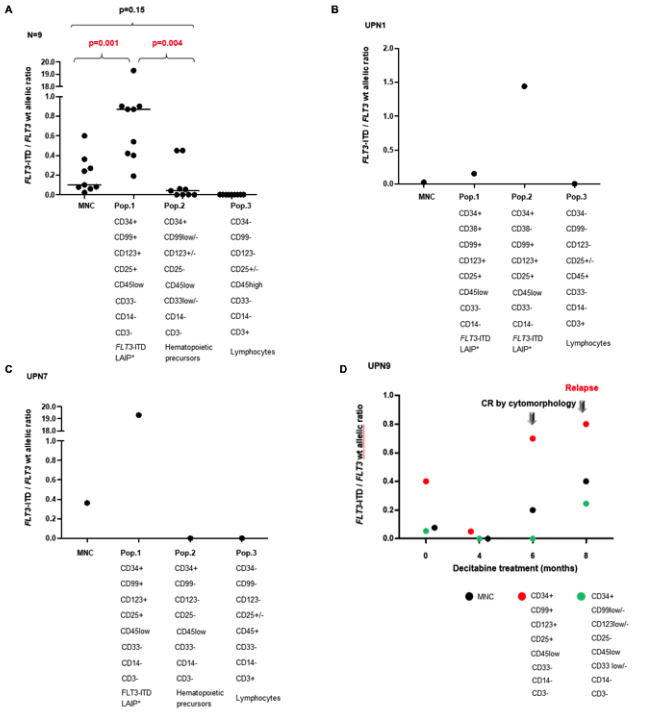

Figure 1. FLT3-ITD allele ratio analysed in several BM AML samples. (A) FLT3-ITD allelic ratio analysis in 9 AML patients collected at diagnosis and in particular in (B) UPN1, (C) UPN7 and (D) UPN9. The FLT3-ITD mutant allele burden, defined as the ratio of the area under the curve of mutant and wild type alleles, was performed both in total MNC and in several BM high-purified cells. The antibody combination used for cell sorting is indicated. P values were calculated by a paired Student's t-test.

Results: Enrichment of FLT3-ITD positive population, defined as a

significantly higher FLT3-ITD mutation load, was observed within the CD34+ compartment of CD123/CD99/CD25+ cells, as compared to the MNC (p=.001) and the lymphoid/myeloid precursors (CD34+/CD123-/CD25-/CD99-) (p=.004) (Figure 1A). In a patient with 2 different FLT3-ITD clones at diagnosis (UPN1), we identified a LSC population defined by CD34/CD123/CD99/CD25+ with one ITD mutated clone in homozygosity(Figure 1B). Moreover, we investigated whether FLT3-ITD mutation was enriched in the LSCs CD34/CD123/CD99/CD25+/CD38- compartment. In UPN7 the CD34+/CD38- LSCs fraction, characterized by CD123/CD99/CD25 co-expression, represented the dominant FLT3-ITD mutated population in comparison with the CD34+/CD38+(Figure 1C) counterpart. Finally, to trace the clonal evolution of LSCs carrying FLT3-ITD mutation, we sorted different BM cell fractions at diagnosis and during follow-up from UPN9 patient who underwent relapse 8 months after diagnosis. As shown in Figure 1D, an increase of the FLT3-ITD allele burden was detected in the CD34/CD123/CD99/CD25+ subset as compared to the CD34+ cells lacking CD123/CD99/CD25 expression and the total MNC. In this patient, a high FLT3-ITD allele burden in CD34/CD123/CD99/CD25+ fraction was detected already at time of complete morphologic remission and 2 months before hematological relapse.

Conclusions: Our study shows that FLT3-ITD mutation represents a founding clone occurs at an early LSC level as defined by CD34/CD123/CD99/CD25+,CD38- immunophenotype. Prospective studies are needed to assess whether monitoring of this cell subset may allow early identification of patients at higher risk of relapse.

CO078

TARGETING OF NOTCH SIGNALING IN B-CELL ACUTE LYMPHOBLASTIC LEUKEMIA (B-ALL) DRUG RESPONSE

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Introduction: Growing evidence suggests that Notch signaling can modulate drug response in a number of hematological malignancies, such as T-ALL, B-CLL and AML. In B-cell Acute Lymphoblastic Leukemia (B-ALL) we have previously demonstrated that Notch3 and Notch4 support survival of primary B-ALL cells, suggesting a role of Notch signaling in drug response.

Methods: B-ALL cell lines were obtained from ATCC, while B-ALL primary cells were obtained from BM or PB of 45 B-ALL patients. Flow cytometry and western immunoblotting were used to study the expression of Notch receptors and ligands. Cytarabine (Ara-C), Dexamethasone (Dexa) and Doxorubicin (Doxo) were used alone or in combination with Notch modulators, including anti-Notch blocking antibodies, gamma secretase inhibitors (GSIs), and Notch transcription factor inhibitor (SAHM1). Mouse xenograft model of B-ALL was obtained by injecting the B-ALL line RS4;11 in NOD/Shi-scid/IL-2Rγnull mice (NOG). Cell viability was evaluated by Annexin-V/PI and MTT assay; proliferation was assessed through CFSE dilution.

Results: A significant expression of Notch receptors and ligands in B-ALL cells was found, i.e. high expression levels of Notch1, Notch3, Notch4, Jagged2, DLL3 and DLL4. Notably, in primary blast cells deriving from refractory patients the expression of Notch3, Notch4 and Jagged2 was significantly higher as compared to patients achieving complete remission. After chemotherapy, a reduction in expression levels of Notch1-4 was observed as compared to samples collected prior the treatment; we then hypothesized that Notch signaling is critical to drug response in B-ALL. Analysis of B-ALL cells treated with conventional drugs (Ara-C, Doxorubicin and Dexametasone), alone or in combination with Notch signaling inhibitors, showed that GSIs and anti-Notch4 were all able to significantly potentiate drug-induced cell death in B-ALL cells by up-regulating intracellular levels of reactive oxygen species (ROS). These high intracellular levels or ROS were then capable to modulate prosurvival protein levels, such as mTor, Akt, NFκ-B and Erk. *In vitro* observations were successfully translated in NOG mice, where GSI-XII in association with Ara-C significantly lowered leukemic burden in bone marrow of xenograft models of B-ALL, prolonging survival of mice compared to Ara-C alone.

Conclusions: In this study, we have highlighted the prognostic value of Notch expression in B-ALL as well as its critical role in B-ALL cell survival and response to chemotherapy *in vitro* and *in vivo*. Moreover, we have demonstrated that Notch inhibitors can potentiate the Ara-C-mediated clearance of blast cells in bone marrow, thus suggesting a potential usefulness to eradicate minimal residual disease in B-ALL. Overall, our results support further investigation of the efficacy of Notch inhibitors in clinical trials.

CO079

GENOMIC ABNORMALITIES UNDERLYING HIGH PIM1 EXPRESSION IN T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA

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Introduction: PIM1, a member of the PIM family of short-lived serine/threonine kinases, was found to be the site of murine T-cell lymphomas retroviral insertion. Transgenic mice provided the first evidence it is as a putative oncogene, as they developed T-cell lymphomas after a short latency time. Further suggesting that PIM1 plays a pivotal role in T-cell acute lymphoblastic leukemia (T-ALL), *in vitro* experiments have shown that T-ALL cell lines are amongst the most sensitive to PIM1 inhibitors. We aimed to assess PIM1 involvement in a large series of T-ALL.

Methods: In our cohort of 337 T-ALL cases, profiled by CI-FISH, we found a rare t(6;7)(p21;q34)/TRB@-PIM1, in a NOTCH1 positive T-ALL, belonging to the TLX3 group (patient enrolled into the GIMEMA LAL0496). The incidence of PIM1 translocations, was assessed using a PIM1 break-apart FISH assay (genomic clones: G248P83149E2 and RP11-200O9). The involvement of the JAK/STAT pathway was determined by investigating PTPN2 deletion, JAK2, JAK3, ABL1 translocations, and IL7R, JAK1/2/3, and STAT5B hot-spot mutations. PIM1 expression was studied by gene expression profile (GEP, Affymetrix) in a study cohort (n=28), including the index case, and a validation cohort (n=68). To recognize PIM1 over-expressing samples, cases were distributed into quartiles: those belonging to the fourth quartile were classified as PIM1-high cases and their genomic background was evaluated.

Results: JAK/STAT alterations were distributed as follows: PTPN2 deletions were found in 7% of cases. We rarely detected rearrangements of genes encoding for JAK/STAT members which occurred in a mutually exclusive way: TRB@-PIM1 in <1%, JAK3 gain/amplification in 1%, and JAK2-translocations in 4%. Instead, IL7R/JAK/STAT mutations were more frequent and were uncovered in 26% of cases. PIM1 expression was highly variable. The case with t(6;7)(p21;q34)/TRB@-PIM1 belonged to the fourth quartile and showed the highest level of PIM1 expression (study cohort). To define the genetic events underlying PIM1 expression in cases without PIM1 rearrangements, we studied an additional group of cases (validation cohort) classified into the HOXA (n=26), TLX1/3 (n=24), or TAL/LMO (n=18) groups. PIM1 expression correlated with the genomic profile. The highest levels were found in HOXA (p=0.0164) and TLX1/3 (p=0.0132) positive cases, and in cases with abnormalities affecting JAK/STAT members, i.e. JAK1, JAK2, JAK3, IL7R, STAT5A/B, PTPN2, and/or NUP214-ABL1 (p= 0.026).

Conclusions: PIM1 emerged as a "critical" deregulated oncogene in T-ALL with JAK/STAT pathway activation. Although rarely, PIM1 can be directly activated by juxtaposition to the TRB@ enhancer. Regardless of the mechanism/s of activation, PIM1 appears as a suitable target in about 30% of T-ALL; rearrangements/mutations of PIM1 and/or of genes encoding for JAK/STAT members, might be used as predictive biomarker to select T-ALL patients who can benefit from treatment with PIM1 inhibitors.

CO080

MUTATIONAL LANDSCAPE OF RELAPSED ACUTE PROMYELOCYTIC LEUKEMIA

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Introduction: APL is characterized by the PML/RARA oncoprotein, whose role in disease pathogenesis and diagnostics is well established. Additional genetic abnormalities have also been detected in APL both at diagnosis and at time of relapse; however, their biologic and clinical significance remains unclear. Recent reports suggested that mutations in PML and RARA genes play an important role in development of resistance to arsenic trioxide (ATO) and/or all-trans retinoic acid (ATRA). We hypothesize that deciphering the molecular mechanisms involved in APL relapse and resistance may be important for the early identification of patients in need of salvage pre-emptive therapy. Methods: An NGS-based approach was used to analyze a total of 30 APL patients who underwent disease relapse after treatment (15 after ATRA-ATO,15 after ATRA-chemotherapy). The NGS assay included both a 31-target gene panel and a customized assay to identify mutations in PML and RARA. Twenty one patients in continuous complete remission (CCR) at a median follow-up of 42 months (range 12-85), were analysed as controls. Results: A high prevalence of mutations in PML and RARA genes was found in relapse samples (8/30, 27%). Comparing the initial mutation burden of patients who relapsed with those who remained in CCR, we found that the number of concomitant mutations per patient was significantly higher in the former group (median n=2 per patient) compared to controls (median n=1 per patient, P=0.02). Moreover, APL patients with multiple relapses after ATRA-ATO showed a significantly higher number of mutations (median n=2 per patient, P=0.04), and an accumulation of mutations during disease progression. We detected in relapsing patients alterations associated with clonal hematopoiesis such as ASXL1, DNMT3A, JAK2, SRSF2, TET2 and mutations in TP53 (Figure 1A). PML and RARA mutations were mutually exclusive with FLT3-mutations. The identified mutational patterns suggested different models of disease progression. In 8 patients, relapse apparently derived from a mutated subclone present at diagnosis. In particular, we observed the co-occurrence of at least one driver mutation in addition to PML/RARA, which was detected both at disease onset and relapse (Figure 1B). In contrast, in 3 patients, relapse probably emerged from PML-mutated ATO-resistant subclones likely arising under selective pressure of ATO. During clonal selection, these subclones may acquire mutations in genes, such as ASXL1, DNMT3A, JAK2, SRSF2, TET2, TP53, WT1, which confer advantages in self-renewal and proliferation (Figure 1C). Conclusion: The two proposed mechanisms of relapse could explain why PML and RARA mutations in our series were mutually exclusive with driver mutations, such as FLT3-ITD and TKD. Deep molecular sequencing at the time of relapse in APL may help to identify patients with a high mutational burden, and/or with PML-mutated subclones resistant to ATO. This may help informing treatment decisions,including the choice anti-CD33 antibodies or allogeneic stem cell transplantation for cases with unfavorable genetic profile.

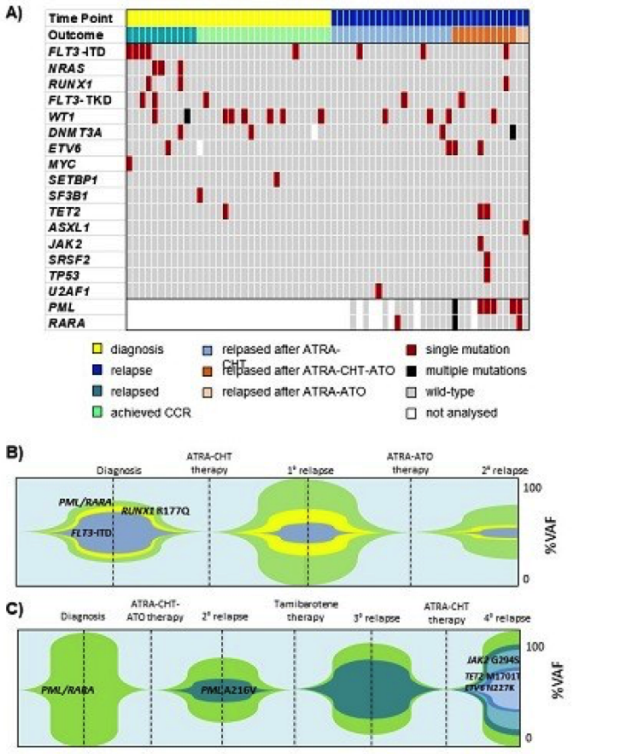

Figure 1. A) Mutational landscape of relapsed and CCR APL B) and C) Different models of disease progression.

Lymphomas

CO081

TP53 MUTATION HAD A NEGATIVE PROGNOSTIC IMPACT IN UNTREATED YOUNG PATIENTS WITH DIFFUSE LARGE B-CELL LYMPHOMA AT HIGH-RISK: A SUB-ANALYSIS OF FIL-DLCL04 STUDY

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Introduction: The Italian phase III randomized study FIL-DLCL04 (Chiappella *et al.*, Lancet Oncol 2017) showed that a consolidation with autologous stem cell transplantation after an abbreviated rituximab-CHOP (R-CHOP) dose-dense chemotherapy (R-HDC+ASCT) compared to a full course of R-CHOP dose-dense chemotherapy, improved failure-free survival (FFS) but not overall survival (OS) in young patients with untreated, high-risk DLBCL. The prognostic role of TP53 is well-known in B-CLL, but it has not yet been established in DLBCL. Aim of this analysis was to correlate TP53 mutations, cell of origin (COO) profile and the presence of biomarkers (MYC, BCL2), with OS and FFS.

Methods: From 2005 to 2010, 399 young untreated DLBCL at poor-risk, were enrolled in FIL-DLCL04 and randomized to receive R-HDC+ASCT in 199 and R-dose-dense in 200 (NCT00499018). TP53 disruption by gene mutation was analyzed by Sanger DNA sequencing. COO classification as germinal center (GCB), activated B-cell (ABC) and unclassified was based on gene-expression profiling using the NanoString research use only lymphoma subtyping test. BCL2 and MYC were studied in immunohistochemistry (IHC); cases were deemed positive if at least 50% and 40% of lymphoma cells were stained with BCL2 and c-MYC antibodies, respectively. BCL2 and MYC translocations, copy gains and aberrations were tested by fluorescent in situ hybridization (FISH). OS and FFS were analyzed; a crude hazard ratio (HR) and an adjusted HR (aHR) for clinical characteristics (treatment, gender, age-adjusted International Prognostic Index, performance status, bone marrow involvement) were calculated.

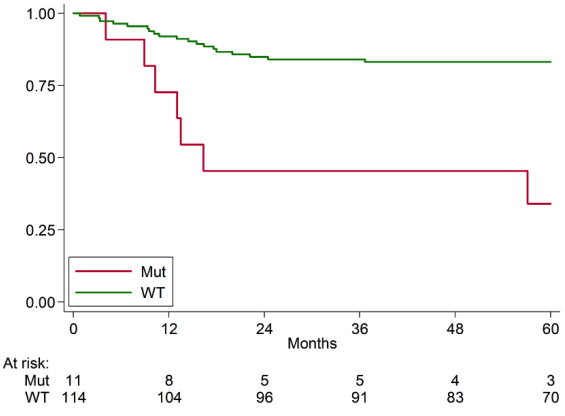

Figure 1.

Results: Of 399 DLBCL patients enrolled in FIL-DLCL04, 125 with tumor block available for subsequent analyses were analyzed for TP53 mutation; no selection bias was observed between the 125 cases and the whole FIL-DLCL04 study population; median age was 51 years and 25 (20%) had bone marrow involvement. Sixty of 125 patients (48%) received R-HDC+ASCT upfront, as for randomization arm. COO was available in 88 patients, 48 (55%) were GCB, 25 (28%) ABC; 26 (23%) were double-expressor (DEL) for MYC and BCL2 in IHC and 11 (10%) double-hit (DHL) for MYC and BCL2 in FISH. Regarding TP53 status, 114 (91%) were wild-type and 11 (9%) mutated. At a median follow-up of 72 months, 5-years FFS for TP53 mutated versus wild-type were 24% (95% CI: 4-52) and 72% (95% CI: 62-79), respectively with a crude hazard ratio (HR) of 3.75 (95% CI: 1.72-8.16), p 0.001, an aHR of 2.21 (95% CI: 0.90-5.46), p 0.085. Five-years OS for TP53 mutated versus wild-type were 34% (95% CI: 9-62) and 83% (95% CI: 75-89), respectively (Figure 1); HR: 5.14 (95% CI: 2.15-12.28), p 0.001, aHR: 3.65 (95% CI: 1.22-10.91), p 0.020.

Conclusions: In this series of young patients with high-risk DLBCL, TP53 disruption by gene mutation identifies a very poor prognosis subgroup with a dismal FFS and OS and identifies patients that may benefit of innovative treatments.

CO082

EARLY STAGE FOLLICULAR LYMPHOMA: BCL2/IGH ANALYSIS AT BASELINE AND FOR MINIMAL RESIDUAL DISEASE MONITORING CAN PREDICT RELAPSE AND DRIVE RITUXIMAB TREATMENT AFTER RADIOTHERAPY

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Introduction: In stage I-II follicular lymphoma (FL), BCL2/IGH+ cells can be detected in the peripheral blood (PB) and/or bone marrow (BM) in a high proportion of cases. We analyzed the prognostic impact of minimal residual disease (MRD) in localized FL and explored the possibility of a MRD-guided rituximab (R) consolidation after standard involved field-radiotherapy (IF-RT).

Methods: Between 2000 and 2016, 67 consecutive patients (pts) with stage I-II FL from a single Institution were investigated for the BCL2/IGH rearrangement by qualitative polymerase chain reaction (PCR) in the PB and BM, and in available lymph nodes (LN). MRD was monitored every 6 months in BCL2/IGH+ pts at baseline. Real-time quantitative PCR (RQ-PCR) and droplet digital PCR (ddPCR) were retrospectively performed in MBR+ cases. All pts were treated with IF-RT (24-30 Gy). From 2005, pts persisting MRD+ after IF-RT received R (375 mg/m2 x 4). The median follow-up is 82 months (17-196).

Results: At diagnosis, 48/67 (72%) pts were BCL2/IGH+ in the PB and BM: 36 (54%) MBR, 6 (9%) mcr, 6 (9%) minor BCL2/IGH rearrangements (Figure 1). Of the 13 evaluable LNs, 11 showed the same marker identified in the PB/BM; 2 cases, negative in the PB/BM, showed a rearrangement only in the LN. The 48 BCL2/IGH+ and 19 BCL2/IGH- cases did not differ in terms of age, stage, grade, FLIPI and primary nodal site. Overall, 23 pts (34%) relapsed, after a median follow-up of 39 months (17-165). Progression-free survival (PFS) was significantly better in pts with absent/low levels (<10-5) of circulating BCL2/IGH+ cells at diagnosis by both RQ-PCR (p=0.029) and ddPCR (p=0.009). IF-RT induced a MRD- status in 50% of cases. R was administered to 19 MRD+ pts after IF-RT and 16 (84%) achieved a MRD- status; 9/16 pts (56.3%) persisted MRD-, and none has so far relapsed (p=0.02). Eight MRD+ pts did not receive R (pre-2005) and 6 (75%) relapsed (p = 0.025). PFS was significantly better for MRD+ pts treated with R compared to untreated MRD+ pts (p=0.011). Overall, of the 39 pts with molecular follow-up, 18 were persistently MRD+ and 21 MRD-: 11 of the 18 (61%) MRD+ pts relapsed, while this occurred only in 4/21 (19%) MRD- pts, who showed a better PFS (p=0.015). The 5-year PFS and OS of the whole cohort are 70% (95% CI: 51%-77%) and 96% (95% CI: 76%–99%), respectively.

Conclusions: In early stage FL, the levels of BCL2/IGH+ circulating cells are a measure of disease spread at diagnosis and bear prognostic

implications. ddPCR might have a higher accuracy compared to RQ-PCR. MRD assessment in the follow-up can predict relapse. IF-RT, the recommended first-line treatment, may not eradicate the disease. R administration in MRD+ pts decreased significantly the risk of relapse and improved PFS. These results support the use of a MRD-guided rituximab consolidation strategy for the management of early stage FL after IF-RT, as explored by the FIL (Fondazione Italiana Linfomi) MIRO' trial (Eudract n. 2012-001676-11).

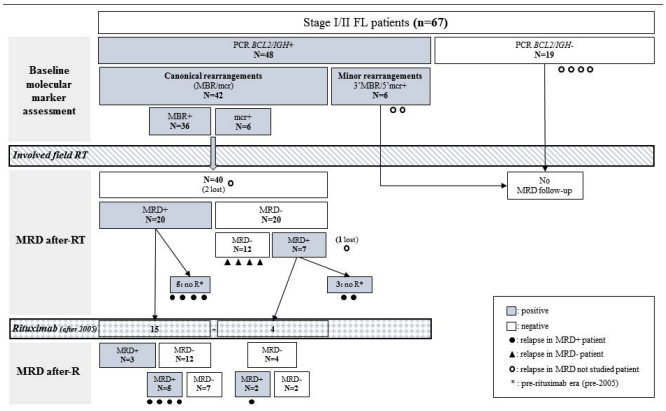

Figure 1.

CO083

MYC-RELATED MICRORNAS SIGNATURES IN NON-HODGKIN B-CELL LYMPHOMAS AND THEIR RELATIONSHIPS WITH CORE CELLULAR PATHWAYS

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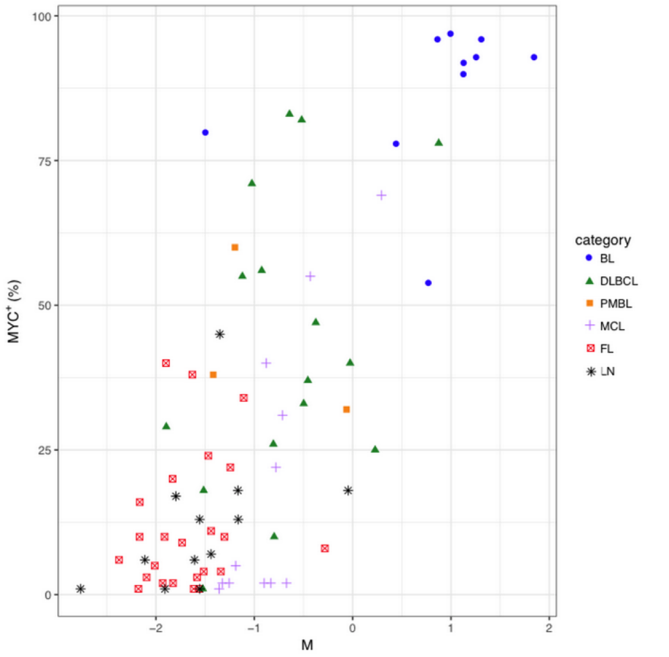
In order to investigate the role of microRNAs in the pathogenesis of different B-cell lymhoma subtypes, we have applied an array-based assay to a series of 76 mixed non-Hodgkin B-cell lymphomas, including Burkitt's lymphoma (BL), diffuse large B-cell lymphoma, primary mediastinal B-cell lymphoma, mantle cell lymphoma (MCL) and follicular lymphoma. Lymphomas clustered according to histological subtypes, driven by two miRNA clusters (the miR-29 family and the miR-17-92 cluster). Since the two miRNA clusters are known to be MYC-regulated, we investigated whether this would be supported in MYC-driven experimental models, and found that this signature separated BL cell lines and a MYC-translocated MCL cell lines from normal germinal center B-cells and other B-cell populations. Similar results were also reproduced in tissue samples comparing BL and reactive lymph node samples. The same series was then quantitatively analyzed for MYC expression by immunohistochemistry and MYC protein levels were compared with corresponding miRNA signatures. A specific metric was developed to summarize the levels of MYC-related microRNAs and the corresponding protein levels. We found that MYC-related signatures are directly related to MYC protein expression across the whole spectrum of B-cells and B-cell lymphoma, suggesting that the MYC-responsive machinery shows predominantly quantitative, rather than qualitative, modifications in B-cell lymphoma. Novel MYC-related miRNAs were also discovered by this approach. Finally, network analysis found that in BL MYC-related differentially expressed miRNAs could control, either positively or negatively, a limited number of hub proteins, including BCL2, CDK6, MYB, ZEB1, CTNNB1, BAX and XBP1.



Distribution of MYC+ cell counts in function of the M parameter in BL, DLBCL, PMBL, MCL, FL and LN samples. MYC expression was assessed by immunohistochemistry with anti-MYC monoclonal Ab in BL, DLBCL, PMBL, MCL, FL and LN samples. MYC+ cells were subsequently counted using the cellSense software. M parameter was calculated for each sample as MYC-upregulated miRNAs minus MYC-downregulated miRNAs. MYC-upregulated miRNAs and MYC-down\regulated miRNAs represent the average level of miRNAs known as upregulated or downregulated by MYC, respectively.

Figure 1.

CO084

THE ROLE OF PERIPHERAL T CELL LYMPHOMA-ASSOCIATED FIBROBLASTS IN CANCER CELL GROWTH

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Introduction: Peripheral T-cell lymphomas (PTCLs) are rare and aggressive types of NHL. Standard front-line therapies include CHOP or CHOP-like chemotherapy, followed by stem cell transplantation. However, a significant proportion of patients is primary refractory or relapses, having a poor prognosis. It is now widely recognised that tumor microenvironment influences cancer cells and acts to sustain tumor progression, aggressiveness and chemoresistance. Among microenvironment components, a central role is played by cancer associated fibroblast (CAFs). Although several studies have addressed the role of CAFs in solid tumors, their role in hematopoietic malignancies have received less attention. Here we report for the first time the isolation and characterization of PTCL-associated CAFs and we describe their role in promoting cell proliferation *in vitro* and *in vivo*.

Methods: A skin biopsy of a cutaneous localization of a PTCL and a PTCL lymph node biopsy were kept in culture. As normal counterpart,

normal fibroblasts were isolated from bone marrow blood samples. Fibroblast-like cells grown out of the tumor biopsies (CAFs) and from the bone marrow blood sample were isolated and cultivated for few passages. Cells were then characterized by flow cytometry, western blot, immunofluorescence and ELISA. The effect of CAFs on PTCL cell growth was assessed by co-culture experiments: the PTCL cell line OCI-Ly12 was cultured in growth medium conditioned either by CAFs or by normal fibroblasts. The role of CAFs was further assessed *in vivo*: we subcutaneously inoculated OCI-Ly12 together with CAFs, OCI-Ly12 and CAFs in NOD/SCID mice. After mice sacrifice, tumor masses were excised for immuhistochemical analyses.

Results: Flow cytometric and biochemical analyses revealed that all cells from our primary cultures express specific fibroblast markers while only CAFs express αSMA, highlighting their activated status. CAFs display an increased rate of proliferation compared to their normal counterparts. In addition, ELISA assays revealed that CAFs are characterized by increased secretion of IL-6 and IL-8 as compared to normal fibroblasts. Co-culture experiments revealed that OCI-Ly12 cell growth is not influenced by CAFs *in vitro*. However we found that CAFs significantly promote tumor growth *in vivo*. Co-injection of PTCL cells (OCI-Ly12) and CAFs give rise to tumor masses characterized by the presence of more vascular structures than those seen in tumor masses from OCI-Ly12 injected alone as demonstrated by immunohistochemical staining with CD31, thereby suggesting that CAFs favour tumor growth by promoting angiogenesis.

Conclusions: Data presented describe for the first time the isolation and characterization of PTCL-derived CAFs. Although these cells do not seem to influence PTCL cell growth *in vitro*, they strongly promote cancer cell growth *in vivo*. This is the first evidence of the role played by the microenvironment in promoting cell proliferation in PTCLs.

CO085

DUAL PI3K δ/γ INHIBITION SUPPRESSES M2 MACROPHAGE POLARIZATION IN HODGKIN LYMPHOMA THROUGH PKM2 DOWNREGULATION

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Introduction: Aberrant activation of the phosphoinositide 3-kinase (PI3K) pathway plays a central pathogenetic role in Hodgkin Lymphoma (HL). Recent data in solid tumors show that pharmacological targeting of PI3K γ isoform in tumor-associated macrophages (TAM) modulates the immunosuppressive microenvironment whereas the δ isoform inhibition results in direct HL cell killing. Since the δ and γ isoforms of PI3K are overexpressed in HL, we propose that the PI3Kδ/γ inhibitor RP6530 might affect the HL tumor microenvironment.

Methods: RNA sequencing (RNA-Seq) and pathway analysis (IPA software) were used to identify differentially expressed genes in HL cell lines (L-540 and KM-H2) treated with RP6530. Serum levels of Thymus- and activation-regulated chemokine (TARC/CCL17) were analyzed by ELISA assay in RP6530-treated HL patients enrolled in a phase 1 study.

Results: Concordantly downregulated genes in L-540 and KM-H2 cells at 6 or 24 h of exposure to RP6530 were involved in cell proliferation, MAPK, JAK/STAT, IL2, IL4/STAT5, glycolysis, HIF1α and MYC signaling, whereas concordantly upregulated genes were involved in cell death, apoptosis and cell cycle deregulation. Consistently 111 downregulated genes across all time points in both cell lines were highly enriched in tumor glycolysis and HIF1α signaling. Among them, we identified 28 hub genes likely to be key drivers in both tumor glycolysis and HIF1α signaling. Pyruvate kinase muscle isozyme M2 (PKM2) was selected as the most important hub gene. In cancer cells PKM2 regulates lactic acid production, an inducer of M2-like macrophages. According to the decreased expression of PKM2 after RP6530 and PKM2 siRNA treatment, we detected a 50% reduction in lactate levels in HL cell lines and downregulation of the expression of the M2 markers CCL17 and CCL22 in the M2-like macrophage population, and a concomitant upregulation of M1 markers. Finally, RP6530 reduced the expression of cytokines/chemokines and angiogenic factors (including, TARC/CCL17, CCL22, CCL5, IL-7, IL-13, and VEGF) known to be secreted by HL tumor cells to promote a pro-tumoral microenvironment. Since TARC/CCL17 is considered a biomarker of disease activity in HL, we prospectively monitored refractory/relapsed HL patients enrolled in a phase 1 trial using RP6530. In RP6530-treated HL patients who experienced complete or partial remission, but not stable or progressive disease, a significant reduction of serum TARC/CCL17 levels was detected after 1 to 12 months of therapy. Additionally, RP6530-responding patients showed a significant reduction of circulating MDSCs further supporting that PI3Kδ/γ inhibition may counteract the immunosuppressive network of HL.

Conclusions: These data demonstrate that RP6530 decreased the expression of PKM2, switching the activation of macrophages from an immunosuppressive M2-like phenotype to an inflammatory M1-like state, suggesting a novel therapeutic opportunity for the treatment of HL patients.

CO086

IMMUNOGLOBULIN KAPPA DELETING ELEMENT (IGK-KDE) REARRANGEMENTS AS POSSIBLE TARGET FOR MINIMAL RESIDUAL DISEASE (MRD) EVALUATION IN MANTLE CELL LYMPHOMA (MCL)

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Background: Minimal residual disease (MRD) assessment is of high clinical relevance in patients (pts) with mantle cell lymphoma (MCL). In mature B-cell malignancies, the presence of somatic hypermutations (SHM) in VDJH rearrangements leads to frequent mismatches between primers, probes and target, thus impairing tumor cells quantification. Alternative targets, such as immunoglobulin kappa-deleting-element (IGK-Kde) rearrangements, are assumed to be free of SHM and could be suitable for MRD detection. In the context of the Fondazione Italiana Linfomi (FIL) MRD Network, we aimed at evaluating the applicability of IGK-Kde rearrangements for MRD quantification in MCL pts by RQ-PCR/digital-droplet-PCR (ddPCR) and at comparing them to IGH and BCL1/IGH rearrangements.

Methods. IGK screening was performed (Pongers-Willemse, 1999) on two cohorts: the first from Turin (24 pts enrolled in the FIL-MCL0208 trial, NCT02354313), the second from Rome (16 outpts). RQ-PCR analyses followed the EuroMRD Consortium guidelines (van der Velden, 2007). The ddPCR was performed as published (Drandi, 2015; Cavalli, 2017).

Results. Of 40 MCL pts, 37 with available diagnostic bone marrow samples (25 IGH+ or BCL1/IGH+, 12 negative for conventional targets) underwent IGK screening: 28/37 (76%) pts resulted IGK-Kde positive, with a target recovery in 8/12 (67%) negative cases. For the ASO primer design, 21/28 (75%) suitable sequences were obtained and in 16/21 (76%) a useful primer for MRD monitoring was obtained. However, the analysis showed a high rate of non-specific amplification of normal mononuclear cell DNA (11/16 cases: 69%), thus hampering the sensitivity of the assay. The MRD RQ-PCR analysis was performed in 30 follow-up (FU) samples from 10 pts: a 100% concordance rate with both BCL-1/IGH and IGH was observed. Finally, a comparative RQ-PCR/ddPCR analysis, carried out on 24 FU samples from 8 pts, showed a 79% (19/24) concordance. In the remaining 5 discordant samples (21%), 4/5 resulted RQ-PCR positive not quantifiable and ddPCR negative, and no clinical relapses were observed, while in 1/5 the ddPCR quantified the disease and the RQ-PCR was negative, but the FU is too short for a clinical correlation.

Conclusions. IGK-Kde rearrangements were found in 76% of MCL, representing a target marker in 67% of IGH/BCL1 negative cases. MRD RQ-PCR monitoring was possible in 57% of cases, showing a 100% concordance with the conventional targets. However, the frequent background amplification affects the sensitivity of the assay, lower in MCL compared to acute lymphoblastic leukemia (Van der Velden, 2002) and in line with multiple myeloma (Puig, 2012). Also in this setting, ddPCR has a good concordance with RQ-PCR and it might help to identify false positive/negative results. From a clinical perspective, we suggest that IGK-Kde MRD monitoring deserves further evaluation in larger series of MCL pts as an additional tool beside the standardized MRD strategies.

CO087

PROTEIN KINASE CK1 ALPHA INHIBITION CAUSES MANTLE CELL LYMPHOMA CELL APOPTOSIS AND SYNERGISTICALLY ENHANCES THE CYTOTOXIC ACTIVITY OF IBRUTINIB

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Introduction: Mantle Cell lymphoma (MCL) is a B-cell malignancy comprising roughly 5-10% of B non Hodgkin lymphomas. MCL patients have been demonstrated to be particularly sensitive to the Bruton Tyrosin kinase (BTK) inhibitor ibrutinib, which by impinging on B Cell Receptor (BCR)-associated signalling events, causes neoplastic B cell apoptosis and proliferation arrest. Nevertheless, refractoriness to ibrutinib may develop portending a dismal prognosis, thus for such cases novel therapeutic strategies are urgently needed. We recently demonstrated that protein kinase CK1α is pivotal for multiple myeloma (MM) cell growth and its inactivation empowers bortezomib and lenalidomide MM induced cytotoxicity. The known CK1α-dependent regulation of growth-promoting cascades, such as the NF-κB and AKT in other tumors, suggests a potential role in MCL downstream the BCR signalling. In this study we analyzed CK1α expression and its role in MCL survival. We analyzed whether CK1α takes part in ibrutinib-induced MCL cell apoptosis and whether blocking CK1α with the chemical inhibitor D4476, or through RNA interference could empower ibrutinib mediated cytotoxicity, through the deregulation of important BCR related survival signalling cascades.

Methods: CK1α expression was analyzed in MCL patients' cells, in cell lines and controls by western blot (WB). CK1α silencing was performed through the generation of anti-CK1α shRNA IPTG-inducible MCL cell clones or through electroporation of double strand CK1α-directed siRNA in MCL cell lines. Cell survival/apoptosis and proliferation were investigated with Annexin V/Propidium Iodide labelling and cytofluorimetric analysis, analysis of PARP cleavage and of pro-apoptotic/pro-survival proteins expression in WB. BCR-dependent signalling events were analyzed by WB. The combination index between ibrutinib and D4476 was calculated through MTT assay.

Results: CK1α was highly expressed in purified primary MCL B cells and cell lines, compared with healthy B cells. Inhibiting CK1α in MCL cells with D4476 or RNAi reduced cell viability, determined apoptosis and proliferation arrest as judged by increased PARP cleavage and caspase activity, by a reduced expression of the pro-survival Mcl1 protein and by deregulation of the cell cycle. CK1α inhibition caused a reduction of activating phosphorylation of RelA/p65 in Ser 536 and of AKT in Ser 473, indicating an important role upstream of critical BCR-dependent signalling pathways. CK1 inactivation together with ibrutinib resulted in a synergic anti-lymphoma effect with a calculated combination index inferior than 1.

Conclusions: Our findings suggest that CK1α is a growth-propelling kinase in MCL, it protects from ibrutinib-induced apoptosis and modulates pivotal growth promoting cascades in MCL cells. CK1 inhibition could represent a rational therapeutic option for MCL and offer the groundwork to design novel combination treatments for this disease.

CO088

INTEGRATED CYTOMETRIC AND IMMUNOHISTOCHEMICAL APPROACH IN DETECTING BONE MARROW INFILTRATION IN NON-HODGKIN LYMPHOMAS: A STUDY OF 388 PATIENTS

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Introduction: Morphological and immuno-histochemical (IHC) analysis of trephine bone marrow biopsies (BMB) is routinely performed during staging of patients with NHL. In this setting, it is not clear whether flow cytometry (FC) study of bone marrow aspirates (BMA) may increase sensitivity and specificity in detecting neoplastic infiltration.

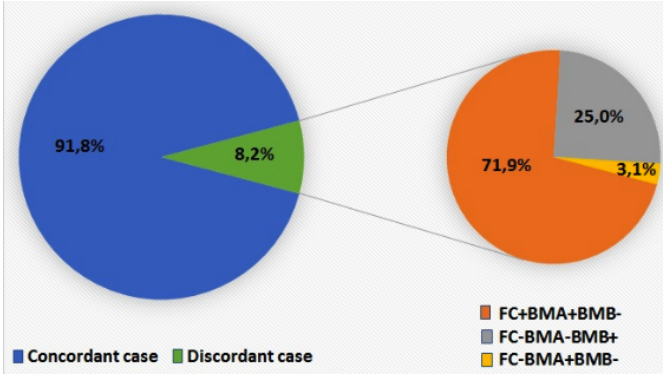

Figure 1.

Methods: Aiming to evaluate the possible diagnostic value of FC on BMA, as compared with BMB, we retrospectively reviewed 388 bone marrow specimens from a consecutive series of patients with SLL/CLL (126), NHL (259) and HCL (3) at diagnosis, in whom these investigations were carried out simultaneously. FC B-cell clonality was assessed by the standard kappa/lambda/CD19 test. Aberrant immune-phenotypes of neoplastic subpopulation were also investigated. A selected panel of monoclonal antibodies was used both for FC and IHC. Concordance was defined as the presence of a positive (in terms of disease detection) or

negative result with FC, BMA cell morphology and BMB (morphology + IHC).

Results: In 356 of 388 specimens (91.8%), there was a concordance among the three investigations. A discordance was detected in 32 cases (8.2%): in 23 of these patients (6% of total population), FC and BMA morphology were both positive, whereas BMB was negative. In 8 of these 32 specimens (2% of total population), FC did not detect lymphoid infiltration concurrently with morphological analysis, although the BMB was considered positive. There was only one case (0,2% of total population) in which both FC and BMB were negative, whereas BMA was reported to be positive. Among discordant samples with FC+BMA+BMB-, 9 corresponded to DLBCL (39%), 7 to MZL (30%), 3 to FL (13%), 2 to MCL (9%), 1 to AITL (4%) and 1 to SLL/LLC (4%). Of 8 cases with FC-BMA-BMB+, 3 corresponded to DLBCL (37,5%), 2 to MZL (25%), 1 to FL (12,5%), 1 to MCL (12,5%) and 1 to AITL (12,5%). A single case with FC-BMA+BMB- was a DLBCL.

Conclusions: In this large retrospective study, most samples showed concordance between morphological and phenotypic studies in BMA and BMB. However, our results also indicated that FC analysis of BMA was able to detect limited bone marrow infiltrations in about 6% of NHL, while in 2% of patients diagnosed as having bone marrow infiltration by BMB, such a localization was not confirmed by FC. The clinical outcome of these cases will be further investigated.

Stem Cells and Growth Factors

CO089

PLASMA CELLS TRANSCRIPTIONAL PROFILE IN SMOLDERING MYELOMA PATIENTS IN RELATIONSHIP TO PROGRESSION TO ACTIVE MULTIPLE MYELOMA

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Introduction: Genomic data support the hypothesis that smoldering myeloma (SMM) patients carry already the genetic alterations that characterized active myeloma (MM) patients and few data are available on the transcriptional profiles of SMM patients in relationship to the progression to MM. In this study, we firstly compared plasma cell (PC) transcriptional profiles from paired samples (SMM progressed to MM), then we investigated transcriptional differences between progressed (P-SMM) and not progressed SMM (NP-SMM) patients.

Methods: 8 P-SMM and 12 NP-SMM with a median percentage of bone marrow plasma cells (BMPCs) of 30% and 14%, respectively were included in this study. SMM was defined according to the IMWG revised diagnostic criteria. The median follow up of the cohort of SMM patients was 49 months. The median time to progression of P-SMM was 14 months and all patients progressed with onset of CRAB features. Primary CD138+ PCs were purified by magnetic beads from bone marrow (BM) aspirate of 8 paired SMM and MM samples and from 12 NP-SMM. High-risk FISH features were detected in 3 out of 8 P-SMM and in 3 out of the 5 NP-SMM patients with enough BMPCs to allow examination. Global expression profiles of protein-coding and non-coding genes were obtained on GeneChip® ClariomD arrays using annotations from Gencode v26. Hierarchical clustering was applied on the most variable coding/non-coding genes across the entire dataset. Rank Product and Gene Set Enrichment Analysis were used for differential and functional analyses. ELISA assays on frozen BM plasma of the same patient cohort were performed.

Results: Hierarchical clustering analysis evidenced paired groupings of the 8 P-SMM with the corresponding MM samples, whereas no markedly agglomerative similarities have been found with/between the NP-SMM cases. Between paired SMM and MM samples, no significant differentially expressed coding/non-coding genes were observed. On the other hand, P-SMM compared to NP-SMM down-regulated antigen processing and presentation and NK-mediated cytotoxicity gene sets, whereas genes associated with proliferation and hyperdiploidy were up-regulated. Specifically, among the 28 most significantly deregulated genes, the Wnt inhibitors (FRZB and DKK1), were up-regulated in relation to progression to MM. This is in line with the recent finding that BM DKK-1 protein level is a new independent factor for progression in SMM. Accordingly, in BM plasma, median FRZB protein levels in NP-SMM patient was significantly lower than P-SMM group (p=0.0038). Finally, 45 non-coding genes, mostly down-regulated (69%), were found modulated in the comparison between P-SMM and NP-SMM cases.

Conclusions: Our data show that the PC transcriptome of SMM patients who progressed to MM did not significantly modify throughout the progression. On the other hand, the upregulation of inhibitors of canonical Wnt signaling by BMPCs distinguished P-SMM from NP-SMM patients.

CO090

PROSURVIVAL AUTOPHAGY IS REGULATED BY PROTEIN KINASE CK1 ALPHA IN MULTIPLE MYELOMA

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Introduction: Multiple Myeloma (MM) is a tumor of malignant plasma cells (PCs) that accumulate in the bone marrow. Both normal and malignant PCs rely on the ubiquitin proteasome system, the unfolded protein response and the autophagic pathway for managing the endoplasmic reticulum stress and ensuring their survival. In particular, autophagy deregulation is maladaptive for MM, since both its inactivation and its hyperactivation may result in cell death. Protein CK1α, a pro-survival kinase in MM, was recently described to be a novel regulator of the autophagic flux in several cancers and a controller of the autophagic transcription factor FOXO3a nuclear exclusion by phosphorylating it on S318/321. Therefore, in this study we aimed at investigating the role of CK1α in the modulation of autophagy in MM.

Methods: CK1α was inhibited with the chemical D4476 and silenced through electroporation of anti-CK1α siRNA or expression of an IPTG-inducible anti-CK1α shRNA. MM cell clones stably expressing the mCherry-eGFP-LC3B fusion protein were generated to monitor the autophagic flux (yellow autophagosomes become red autolysosomes due to the acidic pH-dependent quenching of the eGFP fluorescence). Autophagy related proteins and genes expression in CK1α-inactivated MM cells were investigated by WB and qRT-PCR. Subcellular localization of LC3B and LAMP-2 was evaluated by immunofluorescence.

Results: CK1α inhibition resulted in an impairment in the autophagic flux in MM cells. In this condition, the autophagosome maker LC3B colocalized with the lysosome marker LAMP2, suggesting the formation of autolysosomes; however, in MM cell clones expressing the mCherry-eGFP-LC3B fusion protein green fluorescence was not quenched, therefore an alteration of lysosome acidification resulted in autophagic flux arrest. Moreover, D4476 treatment of MM cells reduced FOXO3a phosphorylation on S318/321, promoting its transcriptional activity in the nucleus. On the contrary, in CK1α silenced MM cells the autophagic flux was correctly triggered, as judged by the accumulation of red vesicles in mCherry-eGFP-LC3B MM clones. However, nuclear localization of FOXO3a was not observed, therefore upregulation of FOXO3a autophagic targets was not achieved.

Conclusions: CK1α inactivation was shown to induce apoptosis in MM cells, however chemical inhibition or silencing of the kinase differently affected the autophagic pathway. D4476-induced cell death could be a consequence of an engulfment of the autophagic/lysosomal machinery due to accumulation of ineffective autophagic vesicles. Differently, upon CK1α silencing, the autophagic flux is correctly triggered, however transcription of autophagic genes sustaining the flux is not achieved. In this latter case, proteins important in the autophagic machinery could be progressively depleted with the consequent autophagy break down, resulting in cell death. Taken together our results suggest a role of CK1α in supporting MM pro-survival autophagy.

CO091

USE OF BOVINE VIRUS AS ALTERNATIVE TOOLS IN MULTIPLE MYELOMA ONCOLYTIC VIROTHERAPY

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Introduction: Oncolytic viruses represent a new class of anti-cancer agents that can selectively infect and lyse cancer cells. Measles virus is the most comprehensively studied oncolytic virus for multiple myeloma (MM) and the first virus to undergo phase I clinical trial investigation. However, the use of human viruses could be limited by the patients' antiviral immune response due to vaccination or natural infection. Bovine Viral Diarrhea Virus (BVDV) is known to be not pathogen for humans and to bind CD46, equally to Measles virus. In this study we investigated the anti-tumoral activity of the BVDV, as an alternative oncolytic strategy in MM.

Methods: We treated the human myeloma cell lines (HMCLs) JJN3, OPM2, and H929 with BVDV or the heat-inactivated virus (hiBVDV) for 24, 48 and 72 hours (h). The infection efficiency was checked by nested multiplex PCR and cell viability by flow-cytometry with 7-AAD staining. Besides, U266 and JJN3 were pre-treated with BVDV for 12h followed by 48h bortezomib (Bor) treatment (2-10nM). Moreover, we infected bone marrow (BM) mononuclear cells (MNCs) obtained from 21 MM patients (11 newly diagnosed, 10 relapsed MM), and 1 with Plasma Cell Leukemia. In this patients' cohort, we studied the BVDV effect evaluating the expression of CD46, CD138, CD14, CD3, CD19 and CD56 by flow-cytometry. Finally to check the possible *in vivo* effect of BVDV, NOD-SCID mice were injected subcutaneously with JJN3 and when plasmacytomas became palpable, BVDV or saline solution was injected intra-tumorally twice a week for 2 weeks. Mice were then sacrificed and analyzed.

Results: Firstly, we found a significant increase of cell death after 48h of infection in the different HMCLs tested (mean±SD% of dead cells in JJN3: BVDV 45±11% vs hiBVDV 16±2.5%, p=0.013, in OPM2: BVDV 43±1.4% vs hiBVDV 28±2.1%, p= 0.015 and in H929 BVDV 29±2.1% vs hiBVDV 11±0.7%, p= 0.007). Conversely, the acute lymphoblastic leukemia cell line 697, did not show a decrease of cell viability, suggesting a BVDV selective effect on MM cells. Interestingly, BVDV pre-treatment significantly increase the cytotoxic effect of 48h Bor treatment in both sensitive and resistant HMCLs ($p<0.0001$). Secondly, we studied the ex vivo effect of BVDV on patients' BM MNCs. The flow-cytometry analysis showed a significant decrease of the percentage of CD138+ cells and median fluorescent intensity after 72h of BVDV infection compared to the control (p= 0.0036). We also observed that the percentage of CD14+ increased after BVDV treatment, while the percentage of CD3+, CD19+ and CD56+ did not change suggesting that the BVDV oncolytic effect was limited to MM cells and potentially associated to monocyte activation. The preliminary *in vivo* results in mice have shown that BVDV treatment reduced tumoral volume compared to controls.

Conclusions: Overall our data indicate a direct oncolytic effect of BVDV on MM cells, suggesting its possible use as novel alternative anti-MM virotherapy strategy.

CO092

INFLAMED BM-MSC HIGHLY ATTRACT TEL-AML1+ PRE-LEUKEMIC CELLS AND PROVIDE A SUSTAINING NICHE FOR THEIR EMERGENCE

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Translocation t(12;21) occurs in utero in stem-progenitor cells (HSPC) but it is insufficient for leukemogenesis, since the TEL-AML1 fusion gene generates a silent B-precursor pre-leukemic clone; additional mutations are thus required for transformation. Infections/inflammation play an important role in leukemogenesis. We previously demonstrated that TGFβ, a cytokine produced during inflammation, limits the proliferation of normal B-precursors favoring the insensitive TEL-AML1+

clone; moreover, TEL-AML1+ Ba/F3 showed alterations in adhesion molecules and SDF1α-directed migration, suggesting possible dysregulated interactions within the BM-niche. MSC are key regulators of HSPC in the niche and posses pro- and anti-inflammatory properties, thus representing a bridge between hemopoiesis and inflammation.

Methods: The murine pro-B cell line Ba/F3 was transfected to generate an inducible TEL-AML1-V5tag protein expression system. Murine BM-derived MSC were isolated and cultured for limited passages. Inflammation was reproduced by treating cells with IL6/IL1β/TNFα. Competitive growth assays were performed by mixing control and TEL-AML1+ Ba/F3. ELISA, annexin-V/7AAD and CSFE stainings were performed following the manufacturer's instructions.

Results: GEP analysis shows that TEL-AML1 affects pathways involved in inflammatory response, cell-cycle/apoptosis and migration. In particular, pre-leukemic cells overexpress the chemokine receptor CXCR2 (MFI: TA=1378±807; ctr=284±167) and migrate more toward its ligand CXCL1 (% migrated cell/input: TA=21.5±6.7; ctr=2.2±1.7). Both murine and human BM-MSC produce CXCL1 and strongly release it during inflammatory stimulation (pg/mL: unstimulated=78±28; inflamed=30162±4760). In accordance, TEL-AML1+ cells are more attracted by inflamed, but not unstimulated, BM-MSCs supernatants (% migrated cell/input: TA=30.2±9.1; ctr=14.3±9.6) in a CXCR2 dependent-manner. Co-culturing control and TEL-AML1+ cells on unstimulated MSC doesn't provide any advantages to the latter, while they increase their percentage respect to controls if inflammatory cytokines are added (fold-increase=1.76±0.27). The advantaging effect depends on BM-MSCs, as inflammatory cytokines do not impact on Ba/F3 cells themselves, and it's mediated by soluble factors. In particular, the complex cytokinic milieu of inflamed MSC-Ba/F3 co-coltures induce an anti-proliferative effect on normal Ba/F3 (fold-increase CSFE-MFI: TA=1.18±0.49; ctr=2.34±0.58) and reduces their viability (fold-increase % ANN-V-/7AAD-: 0.76±0.18). However, neither CXCR2 nor TGFβ blocking revert the effect.

Conclusions: TEL-AML1+ murine B-progenitors are strongly attracted by inflamed BM-MSC in a CXCR2-dependent manner and take advantages from this particular microenvironment. Thus, its blocking could avoid pre-leukemic cells to reach a sustaining niche representing a potential strategy to eradicate TEL/AML1+ pre-leukemic cells.

CO093

FROM FEVER TO IMMUNITY: A NEW ROLE FOR IGFBP-6

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Fever is a fundamental response to infection and a hallmark of inflammatory disease, which has been conserved and shaped through millions of years of natural selection. Although fever is able to stimulate both innate and adaptive immune responses, the very nature of all the molecular thermosensors, the timing and the detailed mechanisms translating a physical trigger into a fundamental biological response are incompletely understood. We analysed the consequence of hyperthermic stress in dendritic cells (DCs), and how the sole physical input is sensed as an alert stimulus triggering a complex transition in a very narrow temporal window. Importantly, our results shed light on significant and specific changes in gene expression and in the metabolic phenotype associated with hyperthermia in DCs. Furthermore, our results support a model based on a thermally induced autocrine signaling, which rewires and sets a metabolism checkpoint linked to immune activation of dendritic cells. Notably, in this context we highlight the novel regulatory functions discovered for IGFBP-6 protein:i) induction of chemotaxis; ii) capacity to increase oxidative burst and degranulation of neutrophils, iii) ability to induce metabolic changes in DCs. In particular, as for the chemotactic effects, we found IGFBP-6 has chemoattractant properties towards monocytes and T cells but not B cells. Recombinant IGFBP-6 increased monocyte migration in a dose-dependent fashion to a maximum of 187±31% of control ($p<0.05$ as compared to cells migrating in the absence of IGFBP-6). T cell chemotaxis was also significantly increased, showing a peak at 4 nM (0.1 μg/ml; 180±29 % of control, $p<0.05$), with a behavior similar to SDF-1. Concentrations of IGFBP-6 lower than 4 nM had no chemotactic activity for T cells. The specificity of the chemotactic effect was shown by the preincubation of IGFBP-6 with an anti-IGFBP-6 antibody, that abolished its chemotactic activity.

Moreover, we focused on the mitochondrial respiratory and oxidative phosphorylation (OxPhos) activity given their pivotal role in the cell bioenergetics and we found that: i) fever-like hyperthermia (3h exposure at 39°C) induces cytokine release in MoDCs; ii) hyperthermia rewires MoDC metabolism by inhibiting mitochondrial OxPhos; iii) the process is linked to interplaying nitric oxide (NO) and reactive oxygen species (ROS) release and mitochondrial (mt) Ca2+ accumulation; iv) antioxidants or mitochondrial Ca2+ uniporter (MCU) inhibition prevents mt-OxPhos inhibition and cytokine release, IGFBP-6 can induce metabolic reprogramming in DCs.

Finally, these findings point to a role of IGFBP-6 in immunity as well as in autoimmune disease; all these novel mechanistic insights could lead to exploit thermal stress related mechanisms in the context of cancer immunotherapy.

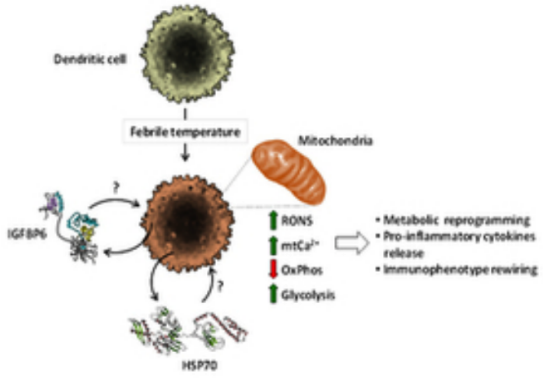

Figure 1.

CO094

CHARACTERIZATION OF MESENCHYMAL STROMAL CELL-DERIVED EXTRACELLULAR VESICLES AND CORRELATION WITH THEIR IMMUNOSUPPRESSIVE PROPERTIES TOWARDS B CELLS

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Introduction: Mesenchymal stromal cells (MSCs) are adult, multipotent stem cells of mesodermal origin. In addition to their stem cell properties, MSCs possess broad immunosuppressive functions influencing both adaptive and innate immune effector cells (IECs). This anti-inflammatory potential is triggered by the release of inflammatory cytokines produced by immune cells in the microenvironment. Indeed, high levels of inflammatory cytokines induce MSCs to become immunosuppressive (primed-MSCs). The immunosuppressive potential of primed-MSCs results from a dynamic interaction between MSCs and IECs mediated by the release of bioactive factors, including extracellular vesicles (EVs). EVs are secreted by many cell types and influence various biological processes, both directly activating cell surface receptors through bioactive ligands and delivering transcription factors, oncogenes, mRNA, and non-coding regulatory RNAs into target cells. In this study, we have investigated the undisclosed molecular mechanisms regulating the immunosuppressive capabilities of EVs derived from primed-

MSCs (primed-EVs) towards B cells.

Methods: We started with a molecular characterization of MSC-derived EVs (MSC-EVs) by high-throughput approaches. We analyzed miRNAs, long-RNAs and proteins differentially expressed in primed-EVs compared to ctrl-EVs. RNA and protein expression profiles were analyzed through RNA Sequencing and Shotgun Mass Spectrometry (MS), respectively. Different in silico approaches were performed to correlate RNA Sequencing and Shotgun MS results and then to evaluate potential pathways involved in immunosuppressive capabilities mediated by MSC-EVs. To functionally validate our findings, we carried out standardized immunological assay using activated primary B cells.

Results: We found 55 proteins, 15 miRNAs, 247 mRNAs, and 80 lncRNAs differentially expressed in primed-EVs compared to ctrl-EVs. Modulated molecules included several proteins and RNA involved in immunological processes, such as MOES, LG3BP, PTX3, and S10A6 proteins, miR-155-5p, miR-497-5p, and IDO1 mRNA. Furthermore, PI3K-AKT signaling pathway and the regulation of actin cytoskeleton were identified and *in vitro* functionally validated as key mediators of MSC-EVs immunomodulatory properties towards activated B cells.

Conclusions: We identified different molecules and pathways potentially responsible for immunoregulatory properties mediated by MSC-EVs. These findings will pave the way for novel therapeutic targets as safer and more useful alternatives to cell-therapy approaches in the fields of inflammatory and autoimmune diseases.

CO095

MEMBRANE DYNAMICS AND FUNCTIONAL EFFECTS ON MYELOMA CELLS AFTER DARATUMUMAB TREATMENT

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Introduction: CD38 is a pleiotropic cell surface glycoprotein with receptorial and enzymatic functions. The molecule is generally expressed at low levels by different hematological and solid tissues: plasma cells score the highest surface levels of CD38 among mature lymphoid cells. CD38 has become the target of therapeutic antibodies in multiple myeloma (MM). Daratumumab (Dara) has been approved as efficient monotherapy or in combination with other anti-myeloma agents. The results obtained are good in patients refractory to standard myeloma therapies. Dara mediates clinical effects through multiple mechanisms, including complement- and antibody-dependent cell cytotoxicity, antibody-dependent phagocytosis, programmed cell death and modulation of enzymatic activities. Promising are Dara immunotherapeutic functions in virtue of its ability to induce cytotoxicity exploiting both arms of innate and adaptive immune responses.

Methods: Microvesicles (MV) were isolated from the culture supernatant of myeloma cells through differential centrifugation steps. MV phenotype was analyzed by flow-cytometry and MV internalization was evaluated by confocal microscopy. NK proliferation, cytotoxicity and IFN-γ production after MV exposure was assessed by flow-cytometry assays. Analyses of gene modulation were performed with NGS.

Results: CD38 engagement at 37°C by Dara on MM cells is followed by a selective polar aggregation of the target molecule in myeloma membranes, with subsequent release of microvesicles (MV) of 100–1,000 nm into the extracellular space. MV released by MM in the bone marrow (BM) niche express functional ectoenzymes (CD38, CD39, CD73, and CD203a), potentially capable of metabolizing both ATP and NAD+ and to produce adenosine (ADO), an immune suppressive molecule. Our results indicate that MV obtained after Dara treatment tend to cluster around (and to be internalized in) NK cells, monocytes and MDSC, cells all expressing IgG Fc Receptors (FcR). NK cells, which apparently disappear in patients during Dara treatment, were selected for testing functional MV-mediated effects. Comparative analysis of the genes modulated after exposing NK cells to the MV/Dara complex were followed by functional *in vitro* experiments. Both sets of results confirmed reduced proliferative ability and NK cell-mediated cytotoxicity. Moreover, in addition to the set of ectoenzymes, the MV phenotype included CD55 and CD59, complement inhibitory receptors, and PD-L1 expression, suggesting that the Dara-driven MV may play a role in the modulation of immune checkpoint pathways.

Conclusions: Dara-driven MV may be a particulate system to influence both the BM niche and the immune response against the malignancy, potentially responsible for Dara resistance in MM.

CO096

ROLE OF TGF-β1/MIR-382-5P/SOD2 AXIS IN THE INDUCTION OF OXIDATIVE STRESS IN CD34+ CELLS FROM PRIMARY MYELOFIBROSIS

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Introduction: Primary Myelofibrosis (PMF) is a myeloproliferative neoplasm characterized by an excessive production of pro-inflammatory cytokines resulting in chronic inflammation and genomic instability. Beside the driver mutations in JAK2, MPL and CALR genes, the deregulation of miRNA expression may also contribute to PMF pathogenesis. To this end, we recently reported the upregulation of miR-382-5p in PMF CD34+ cells.

Methods: In order to unveil the putative role of miR-382-5p in PMF pathogenesis, we performed a gene expression profile of miR-382-5p-overexpressing CD34+ cells by means of Affymetrix HG-U219 Array Strip. To this end, miRNA overexpression was achieved by nucleofection of miR-382-5p mimic using 4D-Nucleofector System (Lonza Group Ltd). TargetScanHuman prediction algorithm 7.0 was used to identify the predicted targets of miR-382-5p.

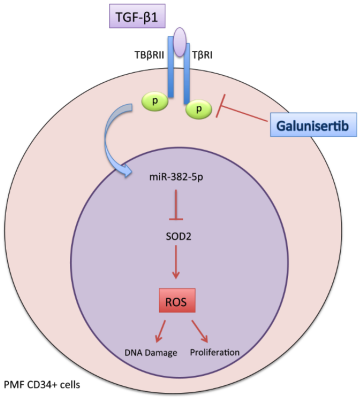

Figure 1.

Results: Among the downregulated genes, we selected the superoxide dismutase 2 (SOD2) as the miR-382-5p most favourable predicted target and we confirmed the miR-382-5p/SOD2 interaction by luciferase assay. We showed that miR-382-5p overexpression in CD34+ cells causes the decrease of SOD2 activity leading to reactive oxygen species (ROS) accumulation and oxidative DNA damage. Interestingly, miR-382-5p inhibition in PMF CD34+ cells restores SOD2 function, induces ROS disposal and reduces DNA oxidation. Since the pro-inflammatory

cytokine transforming growth factor- β1 (TGF-β1) is a key player in PMF pathogenesis, we further investigated whether TGF-β1 could be a potential ROS inducer. Our data show that TGF-β1 treatment enhances miR-382-5p expression by reducing SOD2 activity, then leading to ROS overproduction. Finally, the inhibition of TGF-β1 signalling in PMF CD34+ cells by Galunisertib significantly reduced miR-382-5p expression and ROS accumulation by restoring SOD2 activity.

Conclusions: As a whole, here we showed for the first time that TGF-β1/miR-382-5p/SOD2 axis deregulation in PMF cells is responsible for ROS overproduction that might contribute to enhanced oxidative stress and inflammation. Furthermore, our results suggest that Galunisertib might be an effective therapy to reduce abnormal oxidative stress sustained by TGF-β1 in PMF patients.

POSTERS

Acute Leukemia 1

PO001

BITTER TASTE RECEPTORS ARE EXPRESSED ON ACUTE MYELOID LEUKEMIA CELLS AND THEIR STIMULATION MODULATES LEUKEMIA CELL FUNCTIONS

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Background: Acute Myeloid Leukemia (AML) is a clonal disease sprouting from a rare population of leukemic stem cells. Over the past years, alongside with the identification of disease-specific alleles harbored by the AML clone, increasing interest is gaining the contribution that cell-extrinsic factors have in AML generation and maintenance. In this context, the ability of AML cells to detect changes in the microenvironment is important in responsiveness to environmental fluctuations. Bitter taste receptors (T2Rs) are typical G-protein coupled receptors (GPRs) and are normally found in the oral cavity, where they facilitate the bitter taste. Recent studies showed that T2Rs are widely expressed in myriad of other tissue and have been shown to be involved in physiology of respiratory system, gastrointestinal tract and endocrine system thus suggesting a wider function in "sensing microenvironment". So far, very few data about the expression of TAS2Rs in cancer cells are available. In particular, their expression and function in AML cells has not been investigated.

Methods: Primary AML cells and leukemia cell lines were analyzed for T2R expression (GEP and RealTimePCR). Functional assay (proliferation, clonogenic capacity, migration, metabolism, apoptosis,) were performed after T2R activation by denatonium (DEN), a T2Rs agonist.

Results: In the present work, for the first time, we show that leukemia cell lines OCI-AML3, THP-1, KG1 and AML primary cells expressed several T2R subtypes. T2Rs expression is associated with typical GPR-related downstream targets, including β-gustducin and PLC-β2. Stimulation of leukemia cell lines with DEN, induced intracellular Ca2+ concentration increase, thus demonstrating T2Rs functionality. GEP analysis identified a number of genes significantly modulated by DEN treatment. Specifically, DEN-treated leukemic cells underwent down-regulation of genes involved in positive regulation of cell proliferation, metabolism, migration, and cell-cycle. Whereas genes involved in cell adhesion and DNA repair were up-regulated. Functional assays confirmed molecular data. In particular, depending on the extent of stimulation, T2Rs activation inhibited leukemia cell metabolism and proliferation inducing cell cycle arrest in G0/G1 phase or reduced cell viability inducing apoptosis, as demonstrated by caspase cascade activation and mitochondrial stress induction. Of note, a pronounced inhibitory effect of DEN on leukemia cells motility, both spontaneous and in response to CXCL-12, was observed.

Conclusions: Overall, our data indicate that in AML cells the activation of fully functional T2Rs is associated with quiescence induction and prevention of migration, suggesting a role for microenvironment "bitter" molecules in regulating leukemia cell functions. These results may have implications for the discovery of novel pathways modulating leukemic cells and for the development of a new class of therapeutic molecules.

PO002

THE STRENGTH OF DIGITAL-DROPLET-PCR (DDPCR) AND NEXT-GENERATION-SEQUENCING (NGS) DETECTION IN ACUTE LYMPHOBLASTIC LEUKEMIA (ALL) SAMPLES DEFINED POSITIVE NOT QUANTIFIABLE (PNQ) BY REAL-TIME-QUANTITATIVE-PCR (RQ-PCR)

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Background: Minimal residual disease (MRD) is the strongest prognostic factor in both children and adults with acute lymphoblastic leukemia (ALL). Currently, it can be detected by molecular methods based on real-time-quantitative-PCR (RQ-PCR) that use leukemia-specific or patient-specific molecular markers. Digital-droplet-PCR (ddPCR) and next-generation-sequencing (NGS) represent advanced molecular methods that have the potential to overcome some limitations of standard approaches and potentially provide additional benefits. We analyzed ALL follow-up (FU) samples with a MRD level ≤10-4 by RQ-PCR, ddPCR and NGS in order to highlight discrepancies and to define the discriminating power of the new Methods:

Methods: Eleven newly diagnosed adult ALL patients, 5 enrolled in the GIMEMA LAL 1913 protocol and 6 in the GIMEMA LAL 1308 protocol, and their 23 FU bone marrow (BM) samples were studied. RQ-PCR analyses followed the EuroMRD Consortium guidelines (van der Velden, 2007), ddPCR was performed as published (Della Starza, 2016; Cavalli, 2017) and NGS as described (Faham, 2012; Kotrova M, 2017).

Results: Overall, 13/23 (57%) samples have been consistently classified as positive and quantifiable (Q) or negative by both RQ-PCR and ddPCR, while 9/23 were RQ-PCR positive not quantifiable (PNQ), 3 of which were quantifiable by ddPCR and 6 were ddPCR negative. In the remaining FU sample, RQ-PCR was negative and ddPCR was PNQ. These discordant samples were also studied by NGS: in 3 FU samples that were RQ-PCR-PNQ/ddPCR-Q, NGS resulted also Q and 2 subsequent relapses were observed; in 6 FU samples that were RQ-PCR-PNQ/ddPCR-negative, 5/6 resulted also NGS negative and 1/6 resulted NGS-Q, with a sensitivity range 1 logarithm higher than that of other two methods and no recurrence has so far been observed. In the FU sample that was ddPCR-PNQ/RQ-PCR-negative, NGS resulted Q with a MRD level of 10-5 and a relapse was observed.

Conclusions: The principles and limitations knowledge of each technology is of utmost importance for a correct interpretation of MRD results in ALL. At very low MRD levels, it may be difficult to dissect if the signal observed by PCR (not quantifiable) is due to few residual leukemic cells or to a non-specific amplification of normal DNA. The better sensitivity and accuracy of new methods, could be instrumental to univocally define these samples that can be problematic in the clinical practice and potentially be the source of relapse. Among 23 FU samples analyzed, we observed 3 relapsed cases whose FU samples were classified as PNQ or negative by RQ-PCR, but resulted quantifiable by ddPCR and/or NGS. On the contrary, no relapses were recorded in patients whose FU samples were defined RQ-PCR-PNQ, but proved ddPCR/NGS negative. Further studies with a larger number of discrepant cases and a longer FU time will allow to conclusively define the clinical application and implication of these new methods.

P0003

PREVALENCE AND MANAGEMENT OF LATENT TUBERCULOSIS INFECTION IN ADULTS AFFECTED WITH ACUTE LEUKEMIA OR APLASTIC ANEMIA: A RETROSPECTIVE SINGLE CENTER STUDY

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Introduction: Immunocompromised patients (pts) are susceptible to developing active tuberculosis (TB) disease with a relative risk 2-40 times higher than general population. However, scanty information is reported on epidemiology and management of latent TB infection (LTBI) in pts with newly diagnosed acute leukemia or aplastic anemia (AA), especially in Western Countries.

Methods: We retrospectively analyzed the prevalence of LTBI, diagnosed by the interferon-γ release assay (IGRA) Quantiferon®-TB (QFT) test, in 229 HIV-negative pts (median age 54 ys, range 15-80) affected with either acute leukemia (AML 166, ALL 52), receiving intensive chemotherapy or severe AA (11), under immunosuppressive regimens. Most pts (96.9%) were Caucasian, while 8.7% of subjects was foreign born.

Results: Over a period of 8 years (2010-2017), QFT results at diagnosis were available for 221 pts from our Institution. Collectively, QFT test was positive in 19 subjects (8.6%), namely 16 with AML and 3 with ALL, only 2 foreign born. In 32 cases (14.5%) QFT results were initially indeterminate due to low positive control values. However, 8 of these cases were negative on test repetition, so the final rate of indeterminate QFT results was 10.8%. QFT test yielded positive results in 4 of 10 cases with previous TB contact. Among the 19 positive pts, LTBI was diagnosed in 18 cases, whereas a 35-year old Italian woman presented with active pulmonary TB at AML diagnosis, treated with a 4-drug combination while receiving induction chemotherapy. Radiological findings consistent with LTBI, such as lung nodules or calcifications, were observed by chest X-ray or CT scan in 7 of 18 (38.9%) cases. Patients with LTBI were significantly older (median age 64 ys, range 20-73) than those with negative QFT test. Treatment of LTBI based on isoniazid (INH) 300 mg/day with pyridoxine supplementation was administered to 17 patients during intensive chemotherapy, including HSCT for 6 subjects, for a median of 6 months (range 0.2-18). Conversely, one pt did not receive prophylaxis because of weak positivity of QFT test and subsequent early death due to septic shock. Subsequent lung infections occurred in 9 patients with LTBI, without evidence of active TB on cultural and molecular examinations. INH toxicity occurred in 6/17 cases (35.3%), namely hepatotoxicity (3), psychosis (1), skin toxicity (1) and peripheral neuropathy (in 2 pts concurrently receiving vincristine), leading to either temporary or definitive drug withdrawal in 3 and 2 pts, respectively.

Conclusions: In our observational study, we documented a low prevalence of LTBI (8.1%) in acute leukemia pts from an Italian center, using an IGRA as screening tool. None of these cases developed overt TB reactivation despite immunodeficiency and intensive chemotherapy. INH prophylaxis was feasible and effective. However, prospective controlled studies are needed to further investigate efficacy and ideal duration of INH therapy for LTBI in hematologic pts.

P0004

DACTINOMYCIN SHOWS CLINICAL ACTIVITY IN RELAPSE/REFRACTORY ACUTE MYELOID LEUKEMIA WITH NUCLEOPHOSMIN (NPM1) GENE MUTATION

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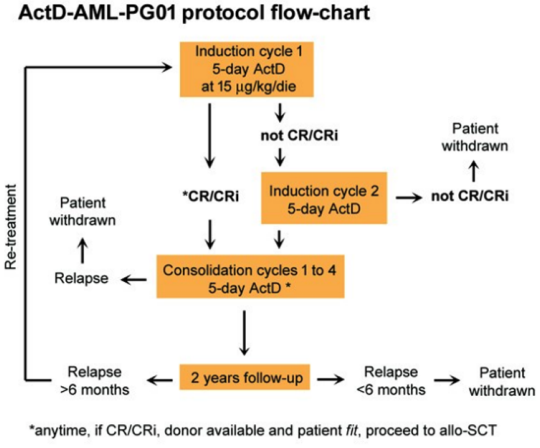
Background: NPM1-mutated AML is the most frequent AML and a new definitive entity in the WHO classification of myeloid neoplasms. A typical feature is the cytoplasmic expression of NPM1, which represents the critical leukemogenic event in this AML. Importantly, the perturbed nucleus-cytoplasmic traffic of the NPM1 mutant with its aberrant accumulation in the cytoplasm leads also to the partial delocalization (from the nucleus to the cytoplasm) of the NPM1 wild-type protein (encoded by the normal allele) through formation of heterodimers with the mutant, thus interfering with the functions of residual wild-type NPM1, including its essential role in the formation and function of the nucleolus.

Hypothesis and Aims: We hypothesized that in the presence of NPM1 mutation, due to the partial depletion of wild-type NPM1 in the nucleolus, leukemic cells would be more 'vulnerable' to drugs inducing nucleolar stress, and reported our first observation that actinomycin D (Dactinomycin, ActD), which triggers nucleolar stress by inhibiting the RNA polymerase I, showed indeed clinical activity in NPM1-mutated B, Brunetti L and Martelli MP. NEJM 2015).

Methods: Here, we have evaluated the clinical efficacy of ActD as single drug in the treatment of NPM1-mutated AML, in a phase II, unicenter, clinical trial (ActD-AML-PG01, EudraCT 2014-000693-18) in 10 refractory/relapse (R/R) AML patients carrying NPM1 gene mutation. ActD was used at the dosage of 15 µg/kg/die for 5 days every 28 days (which defines one cycle) for up to 6 cycles (Figure 1a). Response was defined according to standard criteria.

a



b

Table 1. Characteristics and response rate of R/R *NPM1*-mutated AML patients enrolled in ActD-AML-PG01

Pt. Code	Age (yrs)	FAB	Disease Status	*FLT3* gene status	Previous therapy	Response to Act D	Response duration
UPN001	66	M4	I Relapse/ Resistant	*FLT3*-ITD^{homo}	2-lines CHT	Res	-
UPN002	66	M4	I Relapse/ Resistant	*FLT3*-ITD^{homo}	2-lines CHT	Res	-
UPN003	75	M5	I Relapse	*FLT3*-ITD^{homo}	1-line CHT	Res*	-
UPN004	73	M4	I Relapse	WT	1-line CHT	Early Death	-
UPN005	71	M2	IV Relapse/ Resistant	WT	4-lines CHT	CR (after 2 cycles)	3 mo
UPN006	74	M5b	Resistant	WT	5-Aza (3 cycles)	CR (after 1 cycle)	7 mo
UPN007	53	M4	I Relapse/ Resistant	WT	5-Aza/ Decitabine	Res*	-
UPN008	63	M2	I Relapse	WT	1-line CHT+PBSCT	CR (after 1 cycle)	+24 mo
UPN009	66	M4	I Relapse	*FLT3*-ITD^	1-line CHT	CR (after 1 cycle)	5 mo
UPN010	67	M2	Resistant	WT	1-line CHT	Res*	-

CR: defined by both morphology, flow cytometry and IHC for cytoplasmic NPM1;
*Resistant to a further intensive chemotherapy regimen (UPN007: '3+7'; UPN010: FLAI);
^low ratio (0.012)

Figure 1.

Results: Dactinomycin was relatively well tolerated with major extrahematological toxicity limited to oral mucositis. Anemia, thrombocytopenia and neutropenia were expected during the induction cycle due

to the leukemic bone marrow infiltration. However, no significant hematological toxicity was reported in patients in CR during consolidation therapy. One patient (UPN004), who was colonized by a multiresistant Klebsiella pneumoniae, died during induction phase due to a severe septic shock with multiorgan failure caused by the same microorganism. 4 out of 10 patients (40%) achieved CR, upon either 1 or 2 cycles of therapy (Figure 1b). 3 out of the 4 patients who obtained CR relapsed at 3, 5 and 7 months. One patient (UPN008) underwent haploidentical allogeneic PBSC transplantation at 3 months after CR achievement and is alive at +30 months since CR.

Conclusions: Given the absence of cardiotoxicity, as compared to anthracyclins, and the limited toxicity, either hematological or extrahematological (with the exception of oral mucositis), we believe that actinomycin D might represent a drug to be exploited in the setting of NPM1-mutated AML, and possibly in other AML genotypes. Further either preclinical or clinical studies are warranted to best position this old chemotherapeutic drug in the AML treatment, possibly within combination therapies.

PO005

BROMODOMAIN INHIBITION TARGETS ACUTE MYELOID LEUKEMIA CELLS UNDER HYPOXIA AND ALTERS THE REGULATION OF KEAP1/NRF2 PATHWAY AT TRANSCRIPTIONAL, TRANSLATIONAL AND POST-TRANSLATIONAL LEVEL

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Introduction: The hypoxic microenvironment plays a crucial role in survival and chemoresistance of leukemia stem cells (LSCs) in acute myeloid leukemia (AML). The inhibitor of bromodomain-containing proteins (BETi), which is effective against AML cells in normoxia, targets the transcriptional program controlled by MYC, a crucial gene controlling LSC biology and metabolic functions. The study aims to investigate the role of hypoxia on the selective pressure of BETi treatment on AML cells with different genomic Background:

Methods: AML cell lines (OCI-AML3: NPM1 and DNMT3A mutated, Kasumi-1: t(8;21), HL-60: MYC-amplified, MOLM-13, NOMO-1: MLL-driven, KG-1) were treated for 16/48h with the BETi GSK1215101A (250/500 nM) at 1% or 20% O2 concentration. Downstream analyses were performed on Kasumi-1 and OCI-AML3 cells after 16h of treatment. Gene expression profiling (HTA2.0, Affymetrix) was carried out on actively translated mRNAs isolated by polysome profiling and enrichment analysis was performed by GSEA. The metabolic profile was obtained by Liquid Chromatography-Tandem Mass Spectroscopy (Metabolon).

Results: BETi induced a dose-dependent reduction of cell viability at 48h in AML cell lines under hypoxia (25%-65% decrease at 500 nM) except for HL60. The treatment caused a significant arrest in the G0/G1 phase of the cell cycle in OCI-AML3, Kasumi-1, HL-60 and KG-1 cells ($p<0.05$) and induction of apoptosis in NOMO-1 and Kasumi-1 (40% and 50% AnnexinV+ cells, respectively, $p<0.05$). BETi reduced the translational rate of Kasumi-1 and OCI-AML3 cells, as determined by a decrease of disome-polysome peaks height at polysome profiling. BETi exacerbated MYC suppression under hypoxia and induced downregulation of a MYC-related signature at translational level (NES=-1.6, FDR q=0.06). However, transcriptional and translational levels of NRF2, a target of MYC, were not affected, likely due to the compensatory increase of positive regulators as ARNT and AHR ($p<0.03$). In parallel, expression of the NRF2 inhibitor KEAP1 was reduced by BETi under hypoxia ($p<0.03$), suggesting stabilization of NRF2 protein and activation of the pathway, as confirmed by upregulation of NRF2 targets involved in chaperone, stress and antioxidant response (FTL, FTH1, EPHX) and by increased levels of reduced and oxidized glutathione levels in OCI-AML3 cells ($p<0.01$). Pharmacological inhibition of NRF2 or glutathione biosynthesis failed to potentiate the anti-leukemic effects of BETi.

Conclusions: BETi is effective on AML cell lines under hypoxia, it enhances MYC downregulation, impairs the cell translational rate and highlights novel potential dependencies. BETi-mediated activation of the NRF2 pathway does not function as a cellular mechanism of defence, rather it may contribute to myeloid differentiation of AML cells.

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PO006

ACUTE PROMYELOCYTIC LEUKEMIA (APL) CELLS PROCOAGULANT AND PROADHESIVE PROPERTIES ARE SIGNIFICANTLY AFFECTED BY HEPARINS

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Introduction: Early deaths due to the severe thrombohemorrhagic coagulopathy remain an important cause of treatment failure in APL. Several leukemic cell-dependent mechanisms are involved in APL coagulopathy. Among these, leukemic cell interactions with EC promote localized clotting activation and thrombus formation. Preventing APL cell-EC interactions may represent an approach for hampering excess clotting activation and microthrombi deposition. Experimental evidences suggest that heparins have the capability to affect the tumor cell/EC relationships. In this study we aimed to: 1. evaluate the effects of the LMWH dalteparin and enoxaparin and unfractionated heparin on EC expression of procoagulant tissue factor (TF) and its inhibitor TFPI induced by the APL NB4 cell line; and 2. determine whether the same heparins affect NB4 cell adhesion to EC.

Methods: Human EC of microvascular origin (HMEC-1) were incubated with NB4 conditioned media (CM), or standard IL-1ß (100 IU/ml), in the absence or presence of increasing concentrations of heparins (0.01-1 IU/ml): then, EC TF procoagulant activity and antigen, and TFPI antigen were evaluated. For the adhesion experiments, HMEC-1 were incubated with IL-1ß heparins in the absence or presence of heparins, and thereafter NB4 cell adhesion to EC monolayer was analyzed by microscopy. The effect of heparins on EC surface adhesion molecules (i.e. VCAM-1 and ICAM-1) expression was also tested. The NB4 CM content of soluble mediators (VEGF, FGF-2, IL-1ß, IL-8, TNFα) was measured by ELISA.

Results: Both NB4 CM and IL-1ß significantly increased TF expression by EC. Heparins dose-dependently inhibited the activated EC TF expression, while concomitantly increased the release of TFPI. The analysis of the NB4 CM soluble mediators content showed significant amounts of VEGF and smaller quantities of the other cytokines tested. Furthermore, all heparins significantly ($p<0.05$) counteracted the IL-1ß -induced NB4 cell adhesion to EC monolayer. The LMWH dalteparin was the most effective, inhibiting by 80% NB4 cell adhesion to EC. The reduction of NB4 cell adhesion to EC by heparins was associated with a significant decrease of VCAM-1 and ICAM-1 expression by EC by the same drugs.

Conclusions: This study shows that APL cells can induce the EC prothrombotic phenotype, likely mediated by cytokine release. In the range of clinically relevant doses, heparins can counteract the leukemic-associated prothrombotic stimulus as well as leukemic blast cell adhesion to EC. Our findings demonstrate that heparins, particularly LMWH, can affect in multiple ways the mechanisms of APL-associated coagulopathy and might be good candidates to be tested for thromboprophylaxis in high-risk patients.

PO007

THE NOVEL BRUTON'S TYROSINE KINASE INHIBITOR ARQ531, DISRUPTS SURVIVAL SIGNALING AND TRIGGERS APOPTOSIS IN AML CELLS

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Introduction: Acute Myeloid Leukemia (AML) patient outcome has improved over the last decades. However, resistance develops even to novel therapies and patient overall survival remains low, especially for patients who are not eligible for allogeneic bone marrow transplantation. Therefore, there is an urgent need to overcome the biologic mechanisms underlying drug resistance in AML and facilitate the design of novel approaches. The Bruton's tyrosine kinase (BTK) is emerging as new therapeutic target in a wide range of hematologic malignancies including AML. ARQ531 is an ATP competitive, orally bioavailable, potent and reversible inhibitor of BTK. Herein we present preclinical data on ARQ531 activity in AML cells and in a Patient Derived (PDX) AML mouse model.

Methods: Inhibitory effect of ARQ531 on cell viability was investigated in a panel of AML cell lines as well as primary tumor cells, using both MTT-based assay and FACS analysis over a range of time and doses. The effect of ARQ531 on BTK signaling network and relevant pro-survival pathways was investigated by western blot analysis. Specific transcriptomic profiling of ARQ531-treated AML cells was performed by RNA-Seq compared with untreated cells. Finally, NSG mice engrafted with primary AML cells were used to determine anti-AML activity of ARQ531 *in vivo*.

Results: ARQ531 inhibited AML cell viability with different genetic background (both cell lines and primary cells). Consistent with its effect on cell viability, ARQ531 increased the apoptotic rate of cells tested. Moreover anti-tumoral activity of ARQ531 was not reduced in presence of normal or leukemic mesenchymal stromal cells (MSCs), and, importantly, such treatment showed a good therapeutic window. Mechanistically, ARQ531 treatment specifically blocked BTK and MAPK pathway. A transcriptome profiling analysis of ARQ531-treated cells revealed a reversion of the oncogenic MYC-driven transcriptional program as specific event triggered by ARQ531. As result, Myc-targets inhibition was observed in AML cells treated with ARQ531 compared to Ibrutinib, in a proteomic analysis. Finally, the anti-tumor activity of ARQ531 was determined in AML-PDX model. At 31st day after cell transfer, flow cytometry evaluation of circulating human CD45+ cells in the murine PB revealed a significant reduction of leukemic burden after ARQ531 treatment.

Conclusions: The novel BTK inhibitor ARQ531 is a highly potent kinase inhibitor with promising activity against AML in preclinical models. Our data also support the feasibility of targeting oncogenic MYC-driven translation program by using the safe and novel BTK inhibitor ARQ531.

PO008

AGE, DISEASE STATUS BEFORE BLINATUMOMAB, MRD NEGATIVITY OBTAINMENT, AND BRIDGE TO TRANSPLANT INFLUENCE OUTCOME IN PATIENTS WITH B-ALL: A SINGLE CENTER EXPERIENCE

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Background: Adult B-ALL patients have a dismal prognosis, mainly due to a high relapse rate. Blinatumomab, a bi-specific T engaging CD3-CD19 antibody, has proved efficacy both in MRD positive patients and in relapsed/refractory (R/R) setting. Understanding the most appropriate role of blinatumomab in the therapeutic algorithm of ALL is currently a major issue.

Aim: To evaluate potential predictors of Blinatumomab activity in MRD+ or R/R B-ALL.

Materials and Methods: We retrospectively collected data on 38 patients, who received blinatumomab at Bologna Institute of Hematology "L. and A. Seràgnoli" from January 2014 to September 2017, at the dosage of 9 mcg/d x 7 days, 28 mcg/d x 21 days in 28-days courses. After informed consent was signed, patients were enrolled in two clinical trials (NCT02013167, 5 patients; NCT02000427, 9 patients) or received the drug in a compassionate use program (24 patients).

Results: Median age of the patients was 47 (range 15-72). Philadelphia (Ph) chromosome was detected in 22/38 (58%) patients. All patients received previous lines of therapy (median 3, range 1-7). Before blinatumomab administration, 18 patients were MRD+ (9 Ph+ and 9 Ph-) and 20 patients had a R/R disease (13 Ph+ and 7 Ph-). All patients received at least one course of blinatumomab (median 2, range 1-8). Among MRD positive patients, 10/18 (56%) reached MRD negativity; in 4/18 (22%) cases MRD+ persisted and in 4/18 (22%) patients the disease progressed. Among R/R patients, 9/20 (45%) obtained a CR (7/9 MRD -; 2/9 MRD +). Considering the entire treated population, 19/38 (50%) patients achieved MRD – response, which was obtained after only one course of therapy in 17/19 (89.5%) cases. Furthermore, 12/18 (67%; 10/12 immediately after blinatumomab) MRD + and 4/20 (20%; 3/4 immediately after blinatumomab) R/R patients underwent allo-SCT. MRD + patients who underwent Blinatumomab had more probability to receive SCT than R/R patients (p=.01). Median overall survival (OS) was not reached in MRD + patients; it was 15.2 months (95% C.I: 7.4-23.0) in R/R patients (p=.002). Survival rate at 2-years was 68% and 22%, respectively. In mutivariate models evaluating the role of competitive risk factors on OS, higher age (p=.002; RR 1.03; 95% CI:1.01-1.08) and R/R status before blinatumomab (p=.008; RR 2.9; 95% CI:1.47-5.92) conferred worst prognosis in the general population. The use of blinatumomab as direct bridge to transplant (p=.03; RR .18; 95% CI:.03-.85) conferred a better OS in MRD+ cohort. MRD- response (p=.01; RR .30; 95% CI:.11-.81) conferred a better OS in R/R cohort.

Conclusions: Blinatumomab has a greater efficacy in MRD+ patients, in comparison with R/R setting, as previously reported. In MRD+ ALL, bridging patients to SCT with blinatumomab could be the best therapeutic choice, in order to reach a prolonged survival. In R/R ALL, blinatumomab could confer long term survival to few patients, and MRD-response seems to be an early predictor of good outcome.

PO009

QUANTITATIVE ASSESSMENT OF INDOLEAMINE 2,3-DIOXYGENASE(IDO) EXPRESSION PREDICTS CLINICAL OUTCOME IN ACUTE MYELOID LEUKEMIA PATIENTS AT DIAGNOSIS

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Introduction: Indoleamine 2,3-dioxygenase (IDO) is a key enzyme in the tryptophan metabolism. It catalyzes tryptophan degradation along the kynurenine pathway, thus inducing immune tolerance to tumors. In AML, IDO production was demonstrated to increase Treg cells directly through the conversion of CD25- into CD25+ T cells. IDO expression can be considered a novel mechanism of leukemia escape from immune control and its inhibition may represent a novel antileukemia therapeutic strategy.

Methods: We analyzed 68 patients aged 18 or older, with de novo or secondary AML. IDO expression was evaluated by RT-PCR in blood marrow and peripheral blood samples at diagnosis.

Results: Median age of analyzed patients was 57 years (range 21–76). 59/68 patients (87%) had de novo AML, whereas 9/68 patients (13%) had secondary AML. Risk assessment was available for 61 patients; 13/61 patients (21%) resulted to have a favorable risk AML, 30/61 (49%) an intermediate risk AML and 17/61 patients (30%) were stratified as high-risk AML. Intensive induction chemotherapy was provided for 60 out of 68 patients, 23/68 patients (34%) received allogeneic stem cells transplantation (ASCT) as consolidation therapy. IDO expression in PB samples was between 0.07 and 4272.26 (median 5.60). Conversely, IDO expression in BM samples was between 0.17 and 243.16 (median 1.21). No correlation was found between IDO expression and cytogenetic risk group, de novo or secondary AML, leukocytosis at diagnosis. Among patients who received induction chemotherapy, 35 achieved morphological complete remission (CR), 24 did not respond and 1 patient was not evaluable for response. Among patients undergoing ASCT, we demonstrated that high levels of IDO expression in PB samples negatively correlated with patients' overall survival. Among our study population, we identified two groups of patients with a significantly different outcome in terms of survival, according to IDO mRNA expression: IDO expression < 10 was associated with better overall survival even when adjusted by patients' age (log rank P=0.02). Although the impact on survival of older age and ASCT, IDO expression resulted to be a good predictor of overall survival, independently from patients' age (Cox regression P=0.022) among patients undergoing ASCT.

Conclusions: This work suggests that IDO expression levels can be considered a predictor of AML outcome, independently from other risk factors at diagnosis.

PO011

MYELOID NEOPLASM WITH TRANSLOCATION T(7;8)(Q34;P11) AND FGFR1-TRIM24 FUSION: A NEW CASE WITH UNUSUAL PRESENTATION

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Background: EMS is a rare and aggressive disease driven by rearrangements of the fibroblast growth factor receptor 1 (FGFR1) gene on chromosome 8p11 and one of the 15 so far identified partner genes. The resulting fusion genes encode aberrant tyrosine kinases and activate multiple signal transduction pathways involved in oncogenesis, with some evidence that the partner gene influences the disease phenotype. We report on the second case with a confirmed TIF1(TRIM24)-FGFR1 fusion described to date.

Methods: A 39 yr-old woman was diagnosed in another hospital as AML M4. Bone marrow (BM) karyotype was referred as harboring a t(7;8) and +21, while FISH analysis showed RUNX1 deletion. Despite morphological complete remission (CR) after induction therapy, she shortly relapsed and was transferred to our institution, where cytogenetic and FISH tests [PROBES: dual-color FGFR1 break, BAC RP11-289F17orange (5'TIF1), RP11-288A3green (3'TIF1), RP11-265K5green (3' FGFR1), RP11-359P11orange (5'FGFR1), CEP7 Aqua, CEP8 Aqua] allowed diagnosis of EMS. She successful engrafted after a myeloablative conditioning regimen for allo-HSCT from a matched unrelated donor. Unfortunately, she relapsed six months since transplant and then she was enrolled in a Phase 2 open label monotherapy multicenter study of the new FGFR1 TKI INCBO54828.

Results: BM relapse Karyotype revealed a great clonal eterogeneity: besides the main clone of diagnosis harboring the t(7;8)(q34;p11-12) and +21, other minor sub-clones with different translocation [ie t(3;13), t(6;16), t(9;19),t(1;8)] were found. The breakpoint on 8p11 suggested FGFR1 involvement and further metaphase tricolor FISH tests showed TIF1 (transcriptional intermediary factor 1α) fused with FGFR1. This data was also confirmed by NGS analysis. Of note +21 and RUNX1 deletion were also present since diagnosis. Trisomy 21 seems a non random event associated to disease progression in EMS while RUNX1 deletion has never been reported. The impressive clonal branching observed at relapse suggests that co-operation between RUNX1 and TIF1-FGFR1 in this case acted as trigger of chromosomal instability in the hematopoietic progenitors thus leading to accumulation of multiple genetic changes and aggressive clinical course.

Conclusions: Our finding highlights the need to perform FGFR1 FISH test not only in MPS but also in AML patients with 8p11 translocation. EMS is highly resistant to conventional therapy and allo-HSCT remains the only hope to achieve remission, although unsuccessful in our patient. Therefore, alternative agents, such as FGFR1 inhibitors, are highly recommended, alone or in combination with chemotherapy, in the treatment of these patients. Among a number of concurrent genetic events, RUNX1 haploinsufficiency may play a pivotal role in EMS-AML transformation. The follow up of this case is in progress.

PO012

NOVEL NIPBL GERMLINE PATHOGENIC VARIANT IN A CDLS PATIENT WITH CHILDHOOD ALL: COHESIN INVOLVEMENT IN ALL

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Introduction: Cornelia de Lange syndrome (CdLS) is a rare genetic disorder characterized by pre- and post-natal growth retardation, mental retardation, facial dysmorphism and upper limb abnormalities. The main causes of the disease are mutations in the NIPBL, SMC1A, SMC3, HDAC8 and RAD21 genes, which encode proteins of cohesin complex or associated to it. NIPBL is involved in about 55% of CdLS cases, while the others coexist in 5%. Mutations in the cohesin genes have recently been identified in AML, CML and myelodysplastic syndromes. In the present study, we report the description of the first case of a CdLS pediatric patient who developed precursors B Acute Lymphoblastic Leukemia (BCP-ALL). Furthermore, we investigated the presence of cohesin genes

variants in pediatric ALL patients not affected by CdLS.

Methods: NGS-TC approach on MiSeq platform (Illumina) was evaluated on RNA, by implementing the TruSight Pan-Cancer (Illumina, pre-designed 1385 genes), which included cohesin genes complex, such as NIPBL, SMC1A, SMC3, RAD21, STAG2.

Results: The patient was previously diagnosed as CdLS based on clinical features, and when he was 8-years old, he developed the B-cell precursor ALL and was enrolled into the AIEOP-BFM ALL 2009 study protocol, to high risk treatment group for MRD analysis. At onset, any prognostically relevant cytogenetic abnormality was detected. Through NGS, we identified two mutations in heterozygosity of JAK3 in exon 1 (shared with father) and in exon 16 (shared with whole family in heterozygosity), respectively noted as rs7254346 (benign) and rs3213409 (benign if germline but somatic in ALL/AML). A germline mutation of TP53 exon4, rs1042522, was identified, known as involved in chemotherapeutic resistance (shared with mother in homozygosity). We identified a variant in exon 46 of NIPBL in heterozygosity, a new mutation that causes frameshift, confirmed on bone marrow DNA both at diagnosis, in remission and in buccal smear sample. Both parents and brother are negative, phenotypically normal and not affected by hematological diseases. We further analyzed 86 pediatric BCP-ALL cases after the first CdLS-ALL patient. We assessed potential variants in cohesin genes, detecting 36 variants overall, identifying recurrent known variants in addition to 10 novel variants, mainly affecting NIPBL. However, PCR/Sanger validation is ongoing and already confirmed in three cases out of ten.

Conclusions: It is has been frequently observed an association between germline genetic aberrations and cancer. Indeed, among cancer predisposing syndromes, some have been associated with ALL as for example Down syndrome and Noonan syndrome. Although mutations in cohesins have been found in both adult and pediatric AML, the present study reports the first CdLS pediatric patient with concomitant ALL. The role in leukemogenesis of the new NIPBL gene mutation and in general in leukemia of cohesin genes deserves further investigation.

PO013

IDENTIFICATION OF GENOMIC AND TRANSCRIPTOMIC ALTERATIONS IN THE DNA DAMAGE RESPONSE PATHWAY OF ACUTE MYELOID LEUKEMIA PATIENTS: POTENTIAL TARGETS OF SYNTHETIC LETHALITY APPROACHES

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Introduction: Partner and localizer of BRCA2 (PALB2) plays a key role in the DNA damage response (DDR) and genomic alterations of DDR genes rarely occur in acute myeloid leukemia (AML), while their deregulation at transcriptional level is a known mechanism exploited by leukemic cells to sustain the high genetic instability and to proliferate. We aimed to characterize the role of PALB2 in AML by investigating its genomic alterations and its expression levels, in order to evaluate its potentiality as target of therapies based on a synthetic lethal approach.

Methods: We genotyped 270 AML samples by Single Nucleotide Polymorphism array (SNP 6.0 and Cytoscan HD, Affymetrix). We performed Whole Exome Sequencing (WES, Illumina) of 69 cases (MuTect and Varscan 2.0) and gene expression profiling (GEP, Affymetrix) on bone marrow cells of 7 healthy donors (HD) and 60 AML patients. K-means clustering according to the expression of PALB2 was performed.

Results: We found 11 patients (4%) carrying the CN loss of PALB2 with a minimal common region of 6.6 Kb, involving exons 11-12 (domains of interaction with other DDR genes). Notably, these patients were characterized by the co-occurrence of losses of 5q, 17q11, 16p13-p12 and gain of 21q22. Biologically relevant genes targeted by CNAs were TP53, NF1, BRCA1, STAT3, FANCA, CREBBP, XPO and USP34. Enrichment analysis of differentially altered genes (q<.001) revealed that GO biological processes affected by CNAs included protein folding, apoptosis, mitotic cell cycle, metaphase/anaphase transition, double-strand break repair (p<1e-04). PALB2 was not mutated in our patients and DDR genes such as TP53, BRCA1, BRCA2, CHEK2, FANCE were found mutated at least once. Moreover, PALB2 loss significantly associated with TP53 mutations (p=.015) while KRAS, IDH1/2, TET2 mutations were mutually exclusive. GEP revealed variable PALB2 levels in AML patients (range 52.90-244.37) and its median expression was higher compared with HD (129.26 vs 67.85, respectively; p=.019). We clustered patients according to PALB2 expression and defined 2 groups: H and L with high and low expression levels of PALB2, respectively. Enrichment analysis of differentially expressed genes revealed deregulations of the following GO pathways: mitochondrial translational, regulation of cell proliferation, negative regulation of myeloid cell differentiation and G1/S transition of mitotic cell cycle (p<.013).

Conclusions: We identified alterations in PALB2 and the DDR pathway in a small subgroup of AML patients. Our data open a new scenario in which PALB2 may be a target of therapies in AML: breast cancer patients carrying mutations in PALB2 and BRCA1/2 are candidate for PARP inhibitors treatments, while few clinical trials are available in AML. Therefore, these AML patients may benefit of personalized therapies based on synthetic lethal approaches targeting the DDR pathway.

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PO014

OVERCOME RESISTANCE TO CHEMOTHERAPY BY USING DRUG SENSITIVITY AND RESISTANCE PROFILING IN T-ALL

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Introduction: Drug sensitivity and resistance profiling (DSRP) on primary leukemia samples provide functional informations to assist clinical decision through the identification of actionable targets and biomarkers. Currently the implementation of strong individualized system medicine (ISM) strategies are still a challenge, and clinical studies based on DSRP are critical but anecdotal. Here we present how a DSRP approach informed an alternative treatment in a young patient with refractory T-ALL. T-ALL is a rare but aggressive malignancy originating from T-cell precursors with poor outcome in adult patients and few alternative approaches available in the relapsed/refractory (R/R) setting. Hematopoietic stem cell transplantation (HSCT) plays a pivotal role in R/R patients, but disease status at the time of HSCT has crucial importance to define outcome thus novel strategies are needed to improve overall response in R/R T-ALL.

Methods: in November 2017, a 26 years-old woman with leukocytosis and mediastinum involvement was diagnosed with early T cell precursors ALL. Cytogenetic analysis evidenced a complex karyotype (46,XX[3]46,X,del(Xp),add(10p),add(19p)[12]) and fluorescence *in situ* hybridization (FISH) showed the presence of DDX3X-MLLT10 fusion gene derived by a translocation involving three chromosomes, t(X;3;10)(p11;?;p13). Sequencing analysis confirmed the presence of exon-27 NOTCH1 mutation. The patient was initially enrolled in a standard chemotherapy program (HyperCVAD followed by GIMEMA LAL0904 induction protocol) but displayed a refractory disease. We next decided to test primary blasts assessing ex-vivo the response to multiple small molecules. This library included chemotherapy agents, inhibitors of kinases, cyclins, BET bromodomains, gamma-secretase, and proteasome and anti-apoptotic modulators.

Results: T-ALL blasts showed enhanced sensitivity to Venetoclax and to the proteasome inhibitor Bortezomib. After ethical approval, our patient started a personalized therapy with the anti BCL2 agent Venetoclax (800 mg). We observed a measurable reduction in BM blasts count

(from 60% to 20%) and a decrease of the mediastinum mass. Repeated measurement in T-ALL blasts of sensitivity to Venetoclax during treatment demonstrated a decrease in the ability to achieve a complete cytotoxic effect. To prevent the selection of resistant clones we decided to associate Bortezomib (1.3 mg/m^2 day 1,4,8,11) to the current anti-BCL2 therapy. This combination regimen was well tolerated and led to a further disease reduction compatible with the enrollment of patient in our HSCT program as a well-matched unrelated donor was available.

Conclusion: In leukemia, it is challenging to infer drug activity based solely on genomic data thus the preclinical development assay to rapidly predict sensitivity or response to therapy is critical. Our "N-of-1" approach revealed a way to overcome chemoresistance and suggested a rational well-tolerated strategy in R/R cases of T-ALL.

Chronic Lymphocytic Leukemia and Chronic Lymphoproliferative Disorders 1

PO015

EVALUATION OF ACCURACY OF "CLLFLOW SCORE" BY AN ESTERNAL VALIDATION MULTICENTER STUDY

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Introduction: B-cell chronic lymphoproliferative disorders (B-CLDs) are a group of heterogeneous diseases. A combination of monoclonal antibodies is courrently used for the flow cytometric evaluation. A scoring system based on the surface expression of five markers (CD5, CD23, FMC7, CD79b, and surface immunoglobulins [SmIg]) (Matutes score) was proposed more than 20 years ago. However, the diagnosis of some B-CLDS remains still inconclusive. CD200 has been recently shown to have differential expression in B-CLDs. It has recently reported on the diagnostic performance of a "CLLflow score", calculated by adding the percentages of CD200+ and CD23+/CD5+ B cells and then subtracting the percentages of CD79b+ and FMC7+ B cells. It has been used CD200 instead of SmIg and the percentage expression of each monoclonal antibody on CD19-positive cells. Overall, a CLLflow score >0 is suggestive of chronic lymphocytic leukemia (CLL), while a score ≤0 is consistent with a diagnosis of non-CLL disorder. More recently, our group evaluated the accuracy of another simplified score for the diagnosis of CLL in which only 4 markers are used (CD5, CD23, CD200, and SmIg) showing higher sensitivity and specificity with respect to the "Matutes score".

Methods: We report the results of a retrospective study on 216 patients evaluated for a B-CLDs at our Institutions between September 2009 and April 2018, aiming at comparing the accuracy of "CLLflow score" and our score. Median age at diagnosis was 70 yrs (range 38-97 yrs), and 131 (61%) were male. The diagnosis, according to investigator assessment, was CLL in 161 patients (75%). The non-CLL patients (n=55, 25%) were 32 marginal zone lymphoma (15%), 10 mantle cell lymphoma (MCL) (5%), 8 follicular lymphoma (4%), 4 lymphoplasmocitic lymphoma (1%), 1 hairy cell leukemia (<1%). Sensitivity and specificity for the diagnosis of CLL (vs non-CLL) were calculated (McNemar's test) for the three scores.

Results: The accuracy of "CLLfow score" and our score was not significantly different. In fact, only 5 out 216 patients (2.3%) had a discordant diagnosis when the two scores were applied: 2 CLL patients, 2 MZL patients, and 1 FL patient. On the contrary, the diagnostic accuracy of both "CLLflow score" and our score was significantly different as compared to "Matutes score" (p <0,001 and p0,002, respectively), with a higher number of discordant cases (13 cases for "CLLflow score" vs "Matutes score", and 14 cases for our score vs "Matutes score").

Conclusions: This study confirms the usefulness of both "CLLflow score" and our score for the diagnosis of CLL. Obviously, these findings need further confirmation by means of external validation studies, before being proposed as standardized diagnostic procedures. Overall, data supporting the relevance of CD200 expression evaluation for the diagnosis of CLL are undoubtedly strong, thus suggesting the mandatory inclusion of this marker in the diagnostic panel for B-CLDs.

P0016

PROGNOSTIC IMPLICATIONS OF IGHV GENE REARRANGEMENTS WITH "BORDERLINE" PERCENTAGE OF MUTATIONS IN CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS

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Introduction: The somatic hypermutation (SHM) within IGHV genes classifies chronic lymphocytic leukemia (CLL) patients into two prognostic subgroups: the unmutated (UM) CLL, with adverse prognosis, and the mutated (M) CLL, with a good outcome. Although the 98% cut-off of IGHV identity to the germline counterpart is the approved value to discriminate the two subgroups, for cases with% of mutations is close to the cut-off (i.e. borderline, BL), the prognostic stratification remains controversial (Davis et al, BJH 2016; Rosenquist et al, Leukemia 2017). We analyzed the biologic features and prognosis of BL-CLL within a cohort of 732 untreated CLL patients collected from 1997 to 2017.

Methods: IGHV gene rearrangements were amplified by family-specific VH primers and sequenced using the 3500 Series Genetic Analyzer. The IGHV mutational status was evaluated by the IMGT/V-QUEST tool (version 3.3.0); to determine whether a particular IGHV gene rearrangement belonged to a major stereotyped subset we used the ARResT/AssignSubsets bioinformatics tool (bat.infspire.org/arrest/eri-cll.org/pages/services/tool). Following the recent ERIC guidelines, we reanalyzed old framework region 1 (FR1) BL cases (n=5) with leader primers, to avoid the underestimation of the IGHV SHM rate: 4/5 cases were reclassified as UM-CLL. We identified 57 BL-CLL according to Davis et al, i.e. 97-98.9% IGHV identity (mean: 97.8%) and 34 BL-CLL according to Rosenquist et al, i.e. 97-97.9% (mean: 97.4%). Time-to-first treatment (TFT), calculated from the date of diagnosis to the date of first therapy or last follow-up, was considered as clinical endpoint.

Results: The clinical and biologic features of the 732 untreated CLL patients are reported in Table 1. As expected, UM-CLL (n=351) were significantly different from M-CLL (n=324) for all the biologic parameters reported in Table 1, except for BIRC3 mutation. BL-CLL (n=57) significantly differed from UM-CLL, with a lower frequency of adverse prognostic factors including unfavorable FISH lesions (del11q and del17p) (38% UM vs 18% BL vs 5% M) and NOTCH1 mutations (29% UM vs 12% BL vs 9% M). Regarding the prognostic impact of SHM, UM-CLL showed a shorter TFT than M-CLL ($p<0.0001$), while BL-CLL had a significantly longer TFT compared to UM-CLL ($p<0.0001$) and similar to that of M-CLL. These biologic and prognostic differences held true also when subset #2 cases were excluded from all groups. Moreover the same results were observed when BL-CLL were defined according to the ERIC guidelines (97-97.9% of identity).

Conclusions: This study confirms that UM and M-CLL are two distinct clinical and biologic entities. BL-CLL represent a subgroup with different biologic features compared to UM-CLL and a profile similar to that of M-CLL. Clinically, BL-CLL appear to have a significantly better prognosis than UM-CLL patients in terms of TFT, though the clinical implications of these data need to be conclusively confirmed on larger series of patients.

Table 1. Clinical and biologic features of CLL patients.

	UM-IGHV (n=351)	UM vs BL-IGHV	BL-IGHV (n=57)	M vs BL-IGHV	M-IGHV (n=324)	UM vs M-IGHV
Median age (range)	65.6 (30-87)		65.2 (38-87)		59.1 (27-87)	
Gender (male, female)	187 (70%), 79 (30%)		34 (75%), 11 (25%)		162 (60%), 109 (40%)	
Binet stage (A, B, C)	74 (36%), 103 (49%), 31 (15%)	p=0.0058 (A vs B+C)	20 (63%), 4 (12%), 8 (25%)	p=0.41 (A vs B+C)	155 (71%), 36 (17%), 27 (12%)	p<0.0001 (A vs B+C)
CD38+ (cut-off: 20%)	99/180 (55%)	p=0.004	7/28 (25%)	p=0.09	25/197 (13%)	p<0.0001
ZAP70+ (cut-off: 20%)	125/200 (62%)	p=0.03	12/30 (40%)	p=0.41	67/211 (32%)	p<0.0001
Total cases requiring treatment	339 (97%)	p=0.008	48 (84%)	p=0.3	254 (78%)	p<0.0001
Median TFT, months (range)	17.8 (0-179)	p<0.0001	34.9 (0-219.8)	p=0.26	41.8 (0.3-317.7)	p<0.0001
FISH lesions:	305 available	p=0.0073	45 available	p=0.0071	280 available	p<0.0001
normal	86 (28%)	(del11q and del17p	9 (20%)	(del11q and del17p	90 (32%)	(del11q and del17p
del13q	56 (19%)	vs others)	23 (51%)	vs others)	143 (51%)	vs others)
tris12	46 (15%)		5 (11%)		32 (12%	
del11q	82 (27%)		5 (11%)		9 (3%)	
del17p	35 (11%)		3 (7%)		6 (2%)	
***TP53* status**	26 MUT/200 WT (11%)	p=0.77	3 MUT/33 WT (8%)	p=0.07	5 MUT/235 WT (2%)	p<0.0001
***NOTCH1* status**	77 MUT/187 WT (29%)	p=0.03	4 MUT/30 WT (12%)	p=0.53	15 MUT/153 WT (9%)	p<0.0001
***SF3B1* status**	44 MUT/214 WT (17%)	p=1	5 MUT/26 WT (16%)	p=0.5	9 MUT/158 WT (5%)	p=0.0003
***BIRC3* status**	11 MUT/241 WT (4%)	p=0.06	4 MUT/26 WT (13%)	p=0.1	8 MUT/150 WT (5%)	p=0.81
Major subsets	48/192 (25%)	p=0.0025	9/26 (35%)	p<0.0001	19/118 (16%)	p=0.028
#1	18/48 (37%)	(#1,#2,#8 vs others)	0/9	(#1,#2,#8 vs others)	0/19	(#1,#2,#8 vs others)
#2	2/48 (4%)		8/9 (89%)		4/19 (21%)	
#8	0/48		1/9 (11%)		0/19	
#4	0/48		0/9		1/19 (5%)	

UM: unmutated; M: mutated; BL: borderline; TFT: time-to-first-treatment. Borderline cases were defined as patients with 97-98.9% IGHV identity

PO017

JAK2/STAT3 PATHWAY INVOLVEMENT IN CHRONIC LYMPHOCYTIC LEUKEMIA CELL VIABILITY

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Introduction: Tumor progression in Chronic Lymphocytic Leukemia is regulated by intrinsic events as well as by factors coming from the microenvironment surrounding leukemic cells. Several molecules released by microenvironmental partners signal through JAK (Janus kinases)-STAT (signal transducers and activators of transcription) pathway. The deregulation of JAK2/STAT3 axis may lead to aberrant activation of STAT3 and, as a result, to tumor development in hematopoietic cells. Moreover, once resident in stromal environment, CLL cells are protected from different therapeutic interventions.

Methods: B cells were collected from 23 controls and 53 CLL patients. STAT3 expression and phosphorylation were evaluated by Western Blotting (WB) and Flow Cytometry (FC), and its localization was analyzed by confocal microscopy and subcellular fractionation. Detection of mRNA for STAT3, JAK2 and GAPDH gens was performed by Real Time PCR. Purified cells ($2x10^6$ cells/ml) were cultured, and treated with AG490 (10, 50 and 100 μM) and the STAT3 inhibitor Stattic (5, 7.5, and 10 μM) for 24, 48 and 72h. Experiments with AG490 and Stattic were performed with/without MSCs and with/without Ibrutinib (2.5 μM). CLL and normal B cell viability was tested by FC with Annexin V/PI test.

Results: We demonstrated that STAT3 was highly expressed in malignant B cells with respect to normal B lymphocytes. We also analysed the mRNA levels of JAK2 and STAT3 in normal and CLL B cells and showed that, even if there are no significant differences in JAK2 expression, in pathological lymphocytes there is a higher amount of STAT3 mRNA with respect to normal ones. Moreover, we correlated STAT3 and JAK2 expression with clinical parameters demonstrating that both proteins are homogeneously overexpressed in good vs poor prognosis CLL patients. However, we observed a significant correlation between STAT3 expression levels and their overall survival. We demonstrated that AG490 and Stattic were able to induce a dose-dependent apoptosis in CLL cells and this treatment was able to bypass the environment protection. AG490, targeting JAK2, inhibited the phosphorylation of SHP-1 at Ser591, activating the phosphatase. In turn, SHP-1 activation leads to Lyn Tyr396 dephosphorylation/inactivation. Treatment with Stattic did not affect Lyn and SHP-1 phosphorylation since this inhibitor acts downstream with rispect to AG490. In fact, simultaneous administration of Ibrutinib leads to an increase of apoptosis only in Stattic, but not in AG490 treated cells. This confirms a possible dual role of JAK2 inhibition.

Conclusions: The analysis of expression of JAK2 and STAT3 and the apoptosis due to AG490 and Stattic administration let us to highlight the importance of JAK2/STAT3 axis in the neoplastic cell survival. Furthermore, the correlation with clinical outcome we have demonstrated and the strengthening of Ibrutinib effect could represent a starting point for the development of new therapeutic strategies in CLL.

PO018

HSF1 AS A NEW THERAPEUTIC TARGET IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction: We recently demonstrated that two cytoprotective proteins, the Heat Shock Protein of 70kDa (HSP70) and its transcription factor, the Heat Shock Factor 1 (HSF1), are overexpressed in Chronic Lymphocytic Leukemia (CLL) B cells and correlated to poor prognosis. HSF1 is regulated by a fine balance of activatory/inhibitory phosphorylations mediated by kinases belonging to RAS-triggered pathways. We hypothesize a model by which HSF1 is regulated through different RAS pathways, thus helping to gather information and dissect these networks in CLL. Particularly, the activation of RAS/PI3K/AKT up-regulates HSP70 production, while the activation of the RAS/RAF/MEK/ERK pathway leads to the down-modulation of HSP70 expression.

Considering the pro-survival role played by HSP70 and HSF1 in cancer, in this study we are aimed at testing their druggability in CLL neoplastic B cells. In this context, we used molecules whose activity simultaneously affected the two RAS-mediated pathways, inhibiting AKT and activating ERK to the final purpose of stike HSF1 thus down-modulating HSP70.

Methods: Freshly isolated leukemic B cells from 25 therapy-free CLL patients were cultured in RPMI 1640 supplemented with antibiotics and 2% FBS and treated separately with: 40 μM Resveratrol (a phenol); 10, 20 and 30μM Pterostilbene (a natural analogue of Resveratrol); 10, 50 and 100 μM Triacetyl Resveratrol (a Resveratrol prodrug displaying superior bioavailability than Resveratrol); 5, 10 and 20 μM Honokiol (a poly-phenolic compound whose action resemble that of Resveratrol). Apoptosis was evaluated after 24 hours by Annexin V/Propidium iodide flow cytometry test and by the presence of cleaved PARP in WB. HSP70 and HSF1 expression levels after inhibition were evaluated by Western blotting (WB) analysis in leukemic cells.

Results: We recently found that in CLL, Resveratrol, or molecules with the same mechanism of action, induced apoptosis of neoplastic B cells in a dose-dependent manner. We increased the number of samples analyzed and we observed apoptosis after treatment with: 40 μM Resveratrol (54±20% of living cells vs untreated cells, 70±18%; $p<0.01$, paired Student's t Test); 10μM Triacetyl Resveratrol (65±8% of living cells vs untreated cells, 80±5%; $p<0.01$, paired Student's t Test); 20 μM Honokiol (26±29% of living cells vs untreated cells, 77±8%; $p<0.05$, paired Student's t Test). Pterostilbene showed similar Results: Moreover we found that HSP70 and HSF1 protein levels decreased after the use of these compounds.

Conclusions: HSP70 and HSF1 overexpression and correlation with poor prognosis in CLL patients underline their pivotal role in the regulation of leukemic B cell survival. We succeeded in downmodulating HSP70 expression levels by simultaneously targeting the HSF1 regulation at two different levels. These results allow us to consider HSF1 as a new interesting tool for anti-leukemic therapies.

PO019

IGM MONOCLONAL GAMMOPATY AS INDEPENDENT PROGNOSTIC FACTORS IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA: A RETROSPECTIVE SINGLE CENTER EXPERIENCE

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Background: Chronic Lymphocytic Leukemia (CLL) is an indolent B-cell lymphoproliferative disorder. Different prognostic factors such as IGHV mutation status and chromosomal aberrations as trisomy 12, del11q, del13q or del17p have been detected so far. Nowadays, more recent genetic mutations such as BIRC3, SF3B1, NOTCH1 and TP53 have been discovered stratifying the prognosis and outcome in CLL patients (pts). Considering the costs and the experience of big laboratories, these test are not routinely performed in all medical centers. Scientific literature concerning the presence of IgM, IgG paraprotein and their impact on natural history of CLL pts are controversial and contradictory while more certain seem to be the ones concerning hypogamma/CLL pts.

Aim. The main goal of the study is to establish the prevalence and the outcome of monoclonal IgM/CLL, IgG/CLL and hypogammaglobuline-

mia compared with CLL pts with normal immunoglobulin (Ig) levels.

Methods: Our center collected from our CLL database 404 pts diagnosed from 1999 to 2017 with a baseline assessment of serum Ig, immunofixation, immunophenotype, chromosomal aberrations and clinical features evaluating time to progression (TTP), time to treatment (TTT) and overall survival (OS).

Results: Among 404 pts who met eligibility criteria, 26 pts had IgM/CLL, 33 pts had IgG/CLL, 46 pts had hypogamma/CLL and 299 showed no evidence of paraprotein. Median IgM level was 314 mg/dL in IgM/CLL, median IgG level was 1033 mg/dL in IgG/CLL, median gamma globulin was 7.8% in hypogamma/CLL. Median age was similar among all the groups. The worst time-dependent parameters such as TTP, TTT and OS were identified in the IgM/CLL group. These data probably reflect a more aggressive disease with more than 68% of pts in an advanced stage at diagnosis (Rai B/C).

Summary. This study highlights the frequency of clonal IgM, IgG and hypogammaglobulinemia in CLL patients. Among the 4 groups, IgM/CLL group seems to have the worst outcome which proved to be a negative prognostic marker in newly diagnosed CLL patients.

Table 1. Patients' characteristics.

	IgM/CLL	IgG/ CLL	Hypogamma	Absence of gammopathy
Patients	26	33	46	299
Age (y.o.)	69	63	68	66
Range	(48-82)	(45-86)	(39-89)	(26-85)
Gamma protein (%)	14.4	14.3	7.8	13.1
Range	(7.9-21.5)	(9.1-32)	(5.3-9)	(9-49.5)
IgM level (mg/dL)	314	52	19	54
Range	(31-3300)	(19-330)	(3-300)	(8-1443)
IgG level (mg/dL)	921	1033	535	954
Range	(464-2700)	(471-3939)	(320-1151)	(447-2382)
IGHV				
Mutated	11 (61%)	13 (62%)	17 (52%)	147(63%)
Unmutated	7 (39%)	8(38%)	16 (48%)	88 (37%)
CD38				
Positive	11 (58%)	15(52%)	15 (39%)	43 (16%)
Negative	8 (42%)	14(48%)	23(61%)	231 (84%)
Rai Stage				
A	8 (32%)	18(55%)	21 (45%)	173 (58%)
B	14 (56%)	12(36%)	17 (37%)	111 (37%)
C	3 (12%)	3(9%)	8 (18%)	15 (5%)
TTP (months)	3	19	16	28
TTT (months)	3	22	16	30
OS (months)	49.5	75	72	65

PO020

PROGNOSTIC ROLE OF KARYOTYPE IN CHRONIC LYMPHOCYTIC LEUKEMIA WITH 13Q DELETION

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Introduction: Deletion of 13q (13q-) as isolated change is detected in about 55% of chronic lymphocytic leukemia (CLL) and is usually associated with a favourable outcome, however some patients evolve to a more aggressive disease than expected and novel markers are needed for better prognostication. With the aim to disclose the value of karyotype obtained by using novel mitogen, we defined a new classification based on the combination of IGHV status and cytogenetics in 13q- patients and tested it as predictor of therapy against IGHV alone.

Methods: Our dataset consists of 116 consecutive CLL, collected from 2011-2017 in a single institution, with 13q14.3 (D13S319) deletion as the sole FISH-detected abnormality and studied by cytogenetic analysis with CpG mitogen +IL2 for 72h. Clonal chromosome aberrations were defined following the international guideline in a minimum 20 metaphases (ISCN 2016). IGHV status was established based on gene sequence homology, so >98% of identity was considered non-mutated (U-IGHV) whereas, ≤98% of homology was classified as mutated (M-IGHV). A univariate logistic regression model was applied to evaluate the capability of IGHV status and karyotype to predict the need of therapy.

Results: Among the 116 patients, 62 were males, the median age at diagnosis was 69 years, 85 cases were classified Binet stage A, and 13 Binet B or C. Sixty-four patients were M-IGHV and 26 U-IGHV, whereas the IGHV status was not identifiable or not available (I-IGHV) in 26 patients. Karyotype was normal in 53 cases, it reveals a single abnormality in 30 patients and two chromosome changes in other 17 patients, moreover ≥3 abnormalities (complex karyotype) were present in the last 16 patients. Based on the number of chromosomal aberrations, we defined 2 cytogenetic subgroups: KA including both normal karyotype and cases with a single change, and KB comprising cases with more than 2 abnormalities. Then, we combined IGHV status and karyotype and we re-classified M-IGHV and I-IGHV cases according to the karyotype in unfavorable (UF) or Favorable (F) based on the presence of KB or KA respectively, whereas U-IGHV were kept UF regardless of the karyotype, as expected. Univariate logistic regression analysis showed that the new classification (F/UF) was able to predict patients that will need a treatment (p=0.0013) better than IGHV status (p=0.0228), thus demonstrating that karyotype with ≥ 2 abnormalities worsens the prognosis of M-IGHV and allows to stratify also I-IGHV cases. Moreover, by restricting the analysis to those cases with known IGHV status, the new classification with karyotype returned a p-value (p=0.0037) more significant than considering IGHV only (p=0.0228).

Conclusions: The new classification, based on both karyotype with ≥2 abnormalities and IGHV status, could identify, better than IGHV alone, the subset of patients requiring treatment and supports the added value of cytogenetics to improve the risk stratification in low grade CLL.

PO021

TUMOR MICROENVIRONMENTAL (TME) STIMULI OVERRIDE PEMBROLIZUMAB (PEM) ACTIVITY IN CLL

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Introduction: T cells from CLL patients exhibit defective immunity leading to T cell exhaustion. These functional defects coincide with higher PD1 expression on T cells. Antibodies blocking the PD1 axis are emerging for treatment of hematological neoplasia including CLL. We examined: 1) expression of PD1 and its ligands on B-CLL and autologous T-cells; 2) role of the TME in controlling of PD1 and PDL1/2 expression; 3) anti-PD1 inhibitor PEM (MERCK) effects on the PD1

axis in the TME.

Methods: mRNA of PD1 and PDL1/2 of early-stage CLL patients, belonging to a prospective cohort (n=211, OCLL1, clinicaltrial_ID:NCT00917540) at diagnosis were examined by GEP using the GeneChipVR Gene 1.0 STArray (Affymetrix). CLL clones derived from ex vivo exposure of autologous T-cells with anti-CD3/CD28 Dynabeads (Thermofisher) and IL2 (AAT) were used to mimic the TME and model *in situ* T and B-cell interactions. Flow-cytometry (FC) evaluated cellular phenotype (BD Biosciences). Cytokine levels were measured by FC (BD™Cytometric BeadArray, CBA).

Results: CLL B-cells had higher PD1 mRNA levels than PDL1/PDL2 on GEP, which was confirmed by qPCR (n=20) in 100 samples from an independent cohort of B-CLL patients. Baseline PD1/PDL protein expression showed circulating CLL B-cells had similar levels of PD1 and PDL2, but lower PDL1. PD1 and its ligands in CD3+ cells showed higher PD1 expression, above that of malignant B-cells ($p<0.001$). CD4+PD1 and CD8+PD1 expression was comparable. CLL clones (n=15) exposed to TME stimuli by coculture with AAT, monitored daily (range 2-7d) for T-cell activation (identified as cell-clusters), showed a marked significant ($P<0.001$) upregulation of PD1 ligand mRNA (PDL2>PDL1, 34.3±5.4-fold and 15.4±2.9-fold, respectively) in purified CD19+CD5+ compared to that of PD1 (4.7±0.9-fold). Similarly, cell surface protein expression (n=33) showed both PDLs and PD1 were upregulated compared to baseline in B-cells with PDL2>>PDL1. T-cell subsets by FC detected stronger increase of CD3+, CD4+, and CD8+ cells positive for PDL1, and similar increases for PD1 and PDL2+ in CD4+ or CD8+ cells following AAT coculture. PEM (0.37, 1.11, 3.3, 10.0ug/mL) (n=8) did not influence AAT cluster formation on visual inspection nor PD1/PDL1/2 protein and mRNA expression. PEM (3.3–10 ug/mL) also failed affect IL2, IFNγ, or TNFα levels in supernatants, suggesting an attempt at reactivation of the T-mediated immune response was not possible in this strongly induced cellular context.

Conclusions: We show that TME-derived signals strongly induce gene and protein expression of the PD1 axis in both B- and T-cells from CLL patients. PEM was unable to block PD1 axis protein or mRNA expression or cytokine secretion by T-cells in this context. Although, these unpromising results may be an artefact of the continuous superactivation of T-cells in our model, our data indicate that PEM alone failed to influence both growth of CLL B-cells and restoration of T-cell function. Drug combination studies are warranted. Merck IIS53189 Grant; AIRC Regional Grant n.16695 to FM.

PO022

PI3K INHIBITORS INHIBIT TUMOR MICROENVIRONMENT (TME) REGULATION OF PROGRAMMED DEATH-1 (PD1) AND PDL1/2 IN CHRONIC LYMPHOCYTIC LEUKEMIA (CLL): A POTENTIAL APPROACH TO OVERCOME TUMOR-INDUCED TOLERANCE

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Introduction: Interactions of T cells and macrophages through the PD1 pathway play a crucial role in the CLL TME and ultimately leads to T cell exhaustion and survival and growth of CLL B-cells. The PI3Kδ inhibitor (PI3K-I) CAL101 is approved for patients with relapsed CLL. PI3K signaling is also involved in T-cell activation thus specific PI3K-Is favoring tumor cell-intrinsic activity sparing T-cell functionality are required, such agents include AMG-319 and IPI-145. We examined the expression the PD1 and its ligands, TME-regulation of PD1 axis expression and PI3K-Is effects on the PD1 axis in the TME.

Methods: Early-stage CLL patients (n=110) were prospectively enrolled at diagnosis. Gene (GE) and protein expression were evaluated by qPCR assays (Thermofisher) and flow-cytometry (FC, BD-Biosciences). Activated autologous Tcells (AAT), obtained by *in vitro* exposure of patient T-cells with anti-CD3/CD28 Dynabeads (Thermofisher) and IL2 in co-culture with CLL cells, was used as a model for mimicking the CLL TME. Cultures were monitored until substantial clumping was observed, and then tested for PD1 axis expression by FC. CAL101 (1uM/5uM), AMG319 (1uM), and IPI145 (1uM) (Selleck Chemicals) were added to cell cultures.

Results: Baseline expression of PD1 and ligands from B-CLL samples (n=110) indicated that circulating CD19+CD5+ had similar PD1 and PDL2 protein levels, but limited PDL1, while no significant differences in GE of PD1 or PDLs were observed (n=27). FC (n=106) revealed higher expression of CD3+PD1+ cells, which was significantly higher than in B-CLL cells. CD4+ and CD8+ expressed comparable levels of PD1, whereas CD16+PD1+ expression was lower. Expression of PD1 and PDLs on CD19+CD5+ cells (n=40) showed no significant correlations with prognostic markers. CLL cells after AAT coculture (n=32) showed higher percentage expression by FC of PD1 axis members and PDLs than baseline B-CLL, also confirmed by qPCR GE (n=15). However, T-cell subpopulations had more CD3+, CD4+ and CD8+ cells bearing both PD1 and PDL1, while no substantial changes in CD3+, CD4+ or CD8+/PDL2+ cells occurred after AAT co-culture. Only CD16+PDL1+ cells were upregulated. AAT in presence of PI3K-Is (48h) showed, a reduction of PDL1+ and PDL2+ B-cells by CAL101 (n=9) and AMG (n=5), and more strongly by IPI-145 (n=5), with a similar reduction in PDL1 and PDL2 mRNA (n=7), and a stronger decrease of PD-1, IPI also significantly decreased PDL1/PDL2 mRNA. CAL101 inhibited PD1 expression on CLL cells, CD4+ and CD8+ subsets induced by AAT. AMG had similar effects on the number of CD4+ and CD8+ cells also expressing PD1 and PDL1, but not for PDL2 for both B- and T-cells. CD16 cells showed the greatest reduction in PD1 expression by CAL101 5uM, AMG and IPI, however co-expression of PDLs were also reduced.

Conclusions: TME-derived signals regulate PD1 and PDL1/PDL2 expression in both basal CLL and AAT-cells. PI3K-Is may control immunescape via regulation of PD1 axis expression on neoplastic B- and T-cells in CLL. Gilead Fellowship 2016, AIRC Regional Grant n.16695 to FM.

PO023

PLATELET FUNCTION IN PATIENTS TREATED WITH IBRUTINIB STUDIED USING FLOW CYTOMETRY ON FIXED ACTIVATED PLATELETS

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Introduction: Clinical studies showed that up to 50% of patients treated with Ibrutinib, a Bruton Tyrosine Kinase (BTK) inhibitor, may present a clinically relevant bleeding in the first 3 months of treatment. Ibrutinib-mediated inhibition of the ADP and collagen platelet aggregation pathways has been proposed as a pathogenic mechanism. We used a recently proposed technique, based on flow cytometric analysis of stimulated

platelets to serially evaluate platelet function in patients receiving ibrutinib, in a centralized laboratory facility. This approach has the potential advantage to overcome difficulties related to thrombocytopenia and to improve test standardization.

Methods: Ibrutinib-naïve patients that were consecutively treated in three Institutions between February 2017 and February 2018 were enrolled in this study. Exclusion criteria included concomitant antiaggregant or anticoagulant treatment. Blood samples were collected before treatment and two months after ibrutinib inception. Whole blood collected in citrate was stimulated with ADP/U46619, TRAP or arachidonic acid/epinephrine. After incubation at 37°C for 4-6 minutes, the samples were fixed using PAMFix (Platelet Solutions Ltd, Nottingham, UK) and tested within two weeks using platelet activation marker antibodies, CD62P (platelet surface P-selectin), CD63 (a lysosomal glycoprotein) and PAC-1 (activated GP IIb/IIIa).

Results: We report results of the first 10 patients enrolled in the study. The median age was 66 years with a male/female ratio 8/2. Six patients had CLL, three MCL and one patient had WM. Results were reported in Table 1 (expressed as median fold increase of fluorescence intensity).

Conclusions: This preliminary study suggests that platelet activation by a strong agonist (TRAP) is not impaired by inception of ibrutinib therapy. We observed that platelet activation induced by weak agonists (ADP/U46619 and arachidonic acid/epinephrine) was actually improved by ibrutinib therapy after 2 months. This effect may potentially mitigate the previously described inhibition of platelet activation by the collagen receptor GpVI and can explain the observed lower incidence of bleeding events after the initial 3 months of ibrutinib therapy.

Table 1. Expression of CD62P, CD63 and PAC-1 after stimulation with TRAP, ADP/U46619 and arachidonic acid/epinephrine at baseline (before ibrutinib therapy) and two months after ibrutinib inception (expressed as median fold increase of fluorescence intensity).

	CD62P		P*	CD63		P*	PAC-1		P*
	Baseline	2 months		Baseline	2 months		Baseline	2 months	
Agonist	X (SD)	X (SD)		X (SD)	X (SD)		X (SD)	X (SD)	
TRAP	12.4 (6.1)	18 (13.8)	0.24	5.5 (2.2)	10.5 (17.3)	0.39	1.2 (0.3)	1.2 (0.7)	0.97
ADP/U46619	14.9 (5.3)	29 (18.3)	**0.03**	5.2 (1.0)	10.6 (15)	0.3	1.3 (0.2)	1.5 (1.4)	0.69
arachidonic acid/epinephrine	9.4 (3.6)	28.3 (19.4)	**0.015**	4.2 (1.9)	10.5 (17.9)	0.3	1.0 (0.2)	1.5 (1.8)	0.45

*paired t-test

PO024

IMPACT OF TREATMENT WITH IDELALISIB PLUS RITUXIMAB ON CLINICAL AND CYTOGENETICS PROFILE IN RELAPSE/REFRACTORY CLL: THE EXPERIENCE OF THE UNIVERSITY HOSPITAL OF PARMA

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Background: Idelalisib is an oral inhibitor of p110δ isoform of phosphatidylinositol-3-kinase (PI3Kδ) indicated in association with Rituximab for the treatment of r/r CLL or as first line therapy in the presence of del17p/TP53 mutation (Furman, NEJM 2014). Complex karyotype (CK), defined as the presence of ≥3 chromosomal aberrations, is emerging as high impact feature of CLL on prognosis (Herling, Blood 2016). We conducted a retrospective analysis on clinical efficacy, safety and impact on FISH and karyotype on CLL patients.

Methods: Eleven patients with r/r CLL were treated in our Centre with R-Idelalisib, according to approved schedule, from October 2015 to April 2018. FISH was performed in all patients at the time of chemoimmunotherapy (ChIT), following ChIT and during Idelalisib, while cytogenetics analysis (CG) was performed at the time of ChIT and during Idelalisib. Tumor response was assessed based on the revised International Workshop on Chronic Lymphocytic Leukemia criteria (Hallek, Blood 2018).

Results: Median time from CLL diagnosis to R-Idelalisib was 91 months (range 52-252); median number of previous therapies was 2 (range 1-5). Three out of 11 patients (27.2%) received a prior Btk-inhibitor. FISH analysis at the time of R-Idelalisib shows an unfavorable profile [del(17p), del(11q)] in 10/11 patients (90.9%). CG, before the first therapy, was available for all patients and showed a CK in 2/11 patients (18.1%), an abnormal karyotype in 3/11 (27.2%) and a normal karyotype in 6/11 patients (54.5%). The median OS was 17 months (range 6-30). Nine patients are alive; one patient died for causes not related to therapy, one for progressive disease (PD) after the discontinuation of Idelalisib; three patients discontinued Idelalisib for: (a) intolerance to therapy, (b, c) infective pneumonia; 8 are still on therapy. Median EFS was 3 months (range 1-29); ten patients (90.9%) experienced an AE ≥G3; neutropenia ≥G3 was the most frequent AE. Two patients with CK at the diagnosis went complete remission, although MRD analysis performed in one patient is still positive so far. More interestingly they both went cytogenetic complete response (CyCR) during Idelalisib treatment, although unfortunately one of them died. Furthermore FISH analysis showed an increase of high risk (HR) profile, such as del(17p) and del(11q) over the time frame of ChIT as a result of clonal evolution. Surprisingly, longitudinal monitoring of FISH analysis of HR aberrations revealed a reduction of the allele burden during Idelalisib.

Conclusions. Despite the small population size, our experience confirms that Idelalisib has a good safety profile and is a valid option for HR-FISH profile CLL as well. Idelalisib still represents a good alternative for patients who were intolerant/relapse during another BCR-inhibitor, as previously showed by Mato (Mato, Blood 2016). Furthermore, Idelalisib can induce CyCR in patients with CK and can reduce the clone size in HR-FISH profile CLL.

PO025

LOW-LEVEL TP53 MUTATIONAL LOAD IN CHRONIC LYMPHOCYTIC LEUKEMIA DETECTED BY HIGH-THROUGHPUT NEXT-GENERATION SEQUENCING

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Introduction: Chronic lymphocytic leukemia (CLL) displays a very heterogeneous clinical behavior, therefore prognostic and predictive markers play an important role in disease management. In CLL the worst prognosis is associated with TP53 defects with the affected patients being potentially directed to alternative treatment. Inactivation of the TP53 locus due to del(17p) is frequently associated with mutation(s) on the second TP53 allele. However, TP53 mutations also occur in the absence of del(17p) and are associated with a poor outcome, similar to the disease course observed in del(17p) CLL patients.

Methods: The recent introduction of next-generation sequencing (NGS) has led to the identification of TP53 mutations with a low variant allelic frequency (VAF), below the detection limit of conventional Sanger sequencing, that may be positively selected by the pressure of chemotherapy. Using NGS we performed the analysis of TP53 mutational status of the entire coding region on 260 consecutive CLL patients, referred to our institution for molecular assays. A 30 ng of genomic DNA from peripheral blood samples, was analyzed using Ion Torrent PGM. An amplicon-based library preparation method was performed.

Results: In all cases the minimal coverage obtained was always not less than 100 at any position within the regions of interest (mean coverage 7205 reads) and the number of variant reads for reliable variant calling was at least 10, as suggested by ERIC recommendation. In total, 39 TP53 pathogenic mutations in 34 (13%) patients were found; among them, 35 missense substitutions predominated (89% of detected mutations) and the other 4 mutations were one splice site, one nonsense and two indel. A single mutation was detected in 88% of mutated cases with 12% of mutated patients presenting 2 or more mutations. Moreover, minor TP53-mutated subclones were disclosed in 10/34 TP53 mutated

patients (VAF <15%, range 4-12%), the mutated subclone is the only detected in 7 of them; in these cases only the more sensitive approach allowed to individuate high risk patient resulting more appropriate. All the subclonal TP53 mutation were missense pathogenic substitution included by IARC databases. All mutation were validated by Sanger analysis and the low VAF mutation were validated by a second NGS experiment.

Conclusions: Analysis of TP53 aberrations has been incorporated into routine clinical diagnostics to improve patient stratification and optimize therapeutic decisions. Sanger sequencing is still a recommended approach , but, due to its limited sensitivity may underestimates the TP53 status and misclassified some CLL patients. For this reason , the ERIC 2018 recommendation , introduce the NGS technologies as an applicable one. Subclonal TP53 mutations, individuated by NGS, do not represent passenger events and may have the same negative impact on CLL prognosis. However some advice in the use and interpretation of NGS data may due take in account. This grant was supported by Assessorato alla Salute Regione Sicilia, PSN2013.

Monoclonal Gammopathies and Multiple Myeloma 1

PO026

MARINE DRUGS: SYNTHESIS AND BIOLOGICAL ACTIVITY OF SMENAMIDE-A AND ITS ANALOGUES ON MULTIPLE MYELOMA CELLS

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Marine sponge have proven to be a rich source of novel molecules, often inspiring new strategies in anticancer drug discovery. Smenamide A molecule, isolated from Smenospongia aurea, blocks Calu-1 cell (lung cancer cell line) proliferation. Two stereoisomers, ent-smenamide A and 16-epi-smenamide A, and their analogues have been synthesized. Aiming to investigate *in vitro* the possible therapeutic role of these natural marine molecules in the setting of multiple myeloma (MM), the activity of 16-epi-smenamide A (compound 5) and its analogues (compounds 8, 12-15, 17, 19, 20) was evaluated on two MM cell lines, SKMM1 and RPMI8226. Cell lines were treated with all compounds at increasing concentrations for different time points. Cells treated with DMSO were used as control. Viability was determined using MTS assay and calculated as percentage of viable cells compared with DMSO. Apoptosis and cell cycle studies were performed on RPMI8226 after treatment with compound 10 at 1 and 5μM for 72h using cytometric analysis by labeling cells with annexinV and propidium iodide. Compound 5, tested at increasing concentrations (10-300nM) for 48h, reduced cell viability in both MM cell lines in a dose-dependent way. More than 50% of viability reduction was observed between 30 and 50nM. In fact, EC50 of compound 5 was 44nM in SKMM1 and 24nM in RPMI8226 after 48h of treatment. Compound 8 was used to treat MM cell lines at 0.05, 0.1, 1 and 5μM for 24, 48 and 72h. MTS assay showed that it had no effect on SKMM1, while only a negligible activity was observed on RPMI8226 cell viability. Since compound 5 resulted more active on RPMI8226 cells, its synthetic analogues 12-15, 17, 19 and 20 were tested on this cell line at the same conditions (Figure 1).

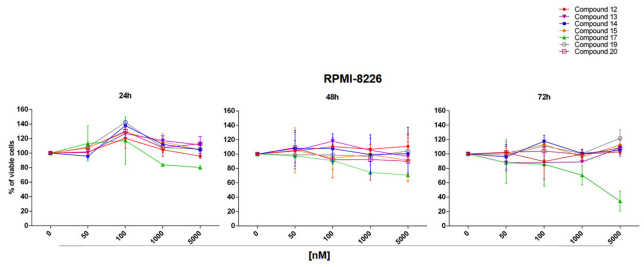

Figure 1.

While compounds 12-15, 19 and 20 had limited activity, compound 17 decreased cell viability by 80% at 5μM after 72h of treatment. EC50 of compound 17 was 1.1μM. Cytometric analysis revealed that compound 17 induced a significant increase of apoptosis in RPMI8226 cells at both 1 and 5 μM after 72h respect to control (5% and 66% of increased apoptosis, respectively). Moreover, it was able to significantly decrease the G0/G1 phase and increase S phase at both concentrations. This preliminary study add new knowledge on the possible role as lead compounds in anticancer drug research of smenamides. Our results showed that compound 5 was active at nM concentration. It was also found that compound 17 exerted a relevant activity, while the truncated compound

8 did not. In particular, compound 17 was able to induce apoptosis and to block cell cycle in MM cell lines. Further studies will be carried out to deepen the structure–activity relationship and to investigate the possible mechanism(s) of action of these compounds.

PO027

INVESTIGATING EXTRACELLULAR VESICLES AS A NEW BIOMARKER IN MULTIPLE MYELOMA AND OTHER MONOCLONAL GAMMOPATHIES

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Introduction: Extracellular vesicles (EVs) are lipid membrane particles of different size and biogenesis, released from normal and neoplastic cells. They have an important role in cell-to-cell cross-talk within a large number of physiological and pathological processes. In particular, EVs from bone marrow (BM) stromal cells may induce proliferation of neoplastic cells and drug resistance in Multiple Myeloma (MM). In turn, EVs from MM plasma cells influence the BM niche, promoting angiogenesis and suppression of immunity. Our group has recently reported that circulating microvesicles (cMVs), an EV sub-micron type purified from MM patient sera, express specific tumor related-antigens, such as CD38 and CD138, and that their number correlate with ISS. In addition, we have also shown that microRNA-155, whose decrease has been associated with MM drug resistance, is reduced in MM serum EVs as compared to healthy controls and other hematological malignancies. Therefore, cMVs could represent a new, non-invasive and promising biomarker, able to avoid more difficult sampling, i.e. from BM. Thus, in a context of liquid biopsy (or, preferably, of "cell biopsy"), we describe here a novel approach to analyze EVs, as a new serum test for monitoring MM.

Methods: In order to carry out a precise and standardized MV analysis, we first proceeded to set up a single step of simple bench centrifugation on serum to obtain a MV enriched pellet. Second, a flow cytometry assay to analyze exactly the most visible MVs in terms of size was defined using beads of known size (between 0.22-1.35 um) and quantity. The MV study was performed by FACSCanto flow cytometric analysis. To analyze lipid membrane particles and not artefacts, we stained MV pellet with protein binding fluorescence dye and anti CD38 antibody. Representative blood samples were collected from MGUS (n=4), smoldering MM (sMM; n=4), MM patients [n=12; de novo(n=4), treatment responders (n=4, and relapsed (n=4) MM], and healthy subjects as controls (n=4).

Results: In considering the cohort preliminary data, we observed a progressive increase of total and CD38 positive MVs in controls, MGUS, sMM and MM. Furthermore, we observed a greater numbers of total and CD38 positive MVs in de novo and progressive MM, as compared to patients in remission phase. More detailed findings regarding the relationship between cMVs and clinical outcome or other prognostic parameters will be presented at the meeting.

Conclusions: A novel method to monitor total and cell -specific MVs as possible new disease biomarker in MM sera was set up. Despite the small number of samples included in this study does not allow to-date any definitive conclusion, we believe that serum CD38+ MVs and ongoing evaluation of their genetic content, could provide a basis for further exploration of their possible role as a non-invasive biomarker in MM.

PO028

IMMUNOPHENOTYPE AND BIOLOGICAL FEATURE OF CD20 POSITIVE IGM NEGATIVE PLASMA CELL NEOPLASMS: A SINGLE CENTER STUDY

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Introduction: The biological significance of CD20 expression on clonal plasma cells (PC) is still uncertain and, except for Waldenstrom Macroglobulinaemia, it is observed in a minority of PC disorders. We have retrospectively analyzed 425 consecutive patients diagnosed in our center to evaluate the immunophenotypic and biological characteristics of CD20+ IgM negative PC neoplasms, defined by >20% CD20+ clonal PC evaluated on the total number of CD38/CD138 positive bone marrow (BM) PC population.

Methods: From January 2006 to January 2018, BM PC population was characterized by multi-color flow cytometry (FC) evaluating the CD19 CD20 CD28 CD38 CD56 CD138 CD45 CD117 surface marker expression. The intra-cytoplasmic (cy) cy-Ig kappa/cy-Ig lambda light chains expression on CD19 CD38 CD56 CD117 CD45 positive PC and on the sub-population of B lymphocytes was also studied to discriminate between clonal and pathological PC and to investigate the presence of clonal BM B lymphocytes. The most common cytogenetic abnormalities as t(11;14), t(4;14), del13q/14, 1p/1q gain, p53 deletion, t(14;16) and the hyper-diploid status were evaluated by FISH analysis.

Results: CD20 expression was documented by FC on malignant PC in 64 cases (15%), 43 multiple myeloma (MM) and 21 monoclonal gammopathy of uncertain significance (MGUS). IgG IgA and no (micromolecular) heavy chains expression was observed in 61% 23% and 16% of cases, with Kappa light chain prevalence (58%). The median age was 66 (40-86) years with a male predominance (56%). Cases were studied at diagnosis, 9 patients were evaluated at disease progression. The CD20 clonal population represented a median of 56% (22-99) of the clonal PC populations, showing a mosaic expression of this marker, which was confirmed in all cases studied during follow up. CD117 CD56 and CD19 expression was observed in 53% 45% and 20% of clonal PC respectively. In 7 cases a bright CD45 expression was documented. The analysis of BM lymphocytes documented immunoglobulin light chain restriction in 15% of cases. By FISH analysis, t(11;14) was the most common cytogenetic findings (23%) with a positive correlation between the percentage of CD20 clonal PC and the percentage of t(11;14) positive cells in the cases analyses after CD138 immunomagnetic beads separation.

Conclusions: CD20 positive PC neoplasms have a distinct immunophenotype characterized by an higher incidence of CD117 and CD19 expression compared to CD20 negative cases. As previously reported, we confirmed our results on the key role of kappa/lambda ratio for the identification of pathological PC at diagnosis as well as for flow-MRD monitoring. Our strategy of FC analysis allowed the identification of CD19+ clonal PC and clonal BM B lymphocytes in 20% and 15% of cases, supporting the involvement of a more immature B-cell precursor in the pathway of the CD20+ PC disorders. An high incidence of t(11;14) and the absence of p53 deletion were observed by FISH analysis.

PO029

CLONAL EVOLUTION AFTER THERAPY REVEALED BY LONGITUDINAL CHROMOSOMAL ABNORMALITIES IN MULTIPLE MYELOMA DISEASE PROGRESSION

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Introduction: The wide heterogeneity in multiple myeloma (MM) includes the presence of high-risk cytogenetic abnormalities (CA) in neoplastic bone barrow plasma cells (BMPC). Some studies showed changes in CA under the effect of chemotherapy. It still remains under debate whether specific therapies are able to target high-risk clones between neoplastic plasma cells and, subsequently, change patients' prognosis.

Methods: We analyzed the CA variations, by FISH analysis, on purified BMPC from Monoclonal Gammopathy of Undetermined Significance (MGUS)/Smoldering Myeloma (SM) to Multiple Myeloma (MM)/Plasma Cell Leukemia (PCL) at diagnosis and progressions/relapses. DNA probes were used for the detection of Rb1 and TP53 deletions, t(11;14)(q13;q32), t(4;14)(p16;q32) and t(14;16)(q32;q23). 1p/1q detection was not available for all patients, since it was implemented in the standard FISH panel from 2012 onward. High risk was defined by the presence of at least del(17p), t(4;14) and/or t(14;16), whereas their absence defined the standard risk.

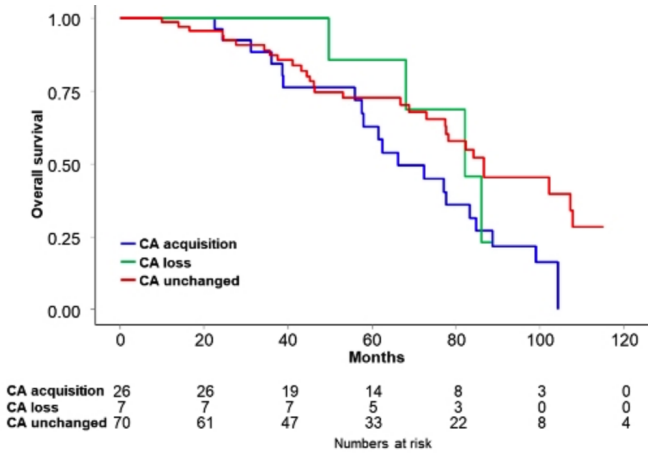

Figure 1.

Results: We analyzed 103 patients (pts): the median follow-up was 60 months (IQR 37-88); males were 54 (53%); median age was 64 years (56-72). According to the International Staging System (ISS), 36% had ISS-I, 41% ISS-II, and 19% ISS-III (4% not available). Twenty-two pts had both MM/PCL and MGUS/SMM diagnostic samples; 55 had MM diagnostic and first relapse samples; all the other pts had at least one subsequent relapse sample. Forty-three (42%) pts received front-line proteasome inhibitor-based therapy; 56 (54%) lenalidomide; 3 (3%) melphalan-prednisone-thalidomide/cyclophosphamide; 32 (31%) autologous stem-cell transplantation; 1 pt did not start any therapy. High-risk CA at diagnosis was present in 28 pts (27%): 17 had del(17p), 11 t(4;14), 4 both CA, no one had t(14;16). Variations in FISH results during disease progression were identified in 33 pts (32%). We classified pts in 3 different populations. The 1st group [CA acquisition] included 25/33 pts (76%) with gain of new CA (Rb1, TP53 deletions, t(4;14), t(14;16) or 1q CA). The 2nd group [CA loss] had 7/33 pts (21%) with loss of a previously identified CA. The 3rd group included 70 patients with no changes. Only 1 patient had gain of t(11;14). We observed the worst survival in the CA-acquisition group, with a median OS of 66 months (95% CI, 58-89 mo). The CA loss group had an OS of 82 mo (95% CI 68-NR mo); the 3rd group of 87 mo (95% CI 78-NR mo; group 1 vs 3, P=0.002; group 2 vs 3, p=0.79).

Conclusions: Our results confirm that clonal evolution occurs as disease progresses after different chemotherapy lines. Moreover, patients who acquired high-risk CA had the poorest prognosis. These findings also highlight the importance of performing FISH analysis both at diagnosis and relapse.

PO030

INNOVATIVE NANOTECHNOLOGY APPROACH FOR HIGH SENSITIVE FISH ANALYSIS IN PLASMA CELL NEOPLASMS WITH LOW BM INFILTRATION

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Introduction: Cytogenetic abnormalities detected by FISH on plasma cell (PC) population selected by anti-CD138 immunomagnetic beads are of primary relevance in classification, risk stratification and management of patients with plasma cell neoplasm. In a significant proportion of cases, however, malignant PC represent a small percentage of bone marrow (BM) cells, hampering the possibility of cytogenetic studies in PC malignancies. A major improvement of FISH analysis for minimally represented sub-populations, such as the circulating tumor cells, has been documented by the application of innovative nanotechnologies. We have evaluated cytogenetic analysis on 15 patients with low percentage of BM clonal PC using the miniaturized device microFIND to evaluate the applicability of this approach in a subset of difficult samples.

Methods: The total percentage of neoplastic PC was evaluated by multicolor flow cytometry characterization on BM samples. FISH analysis using the Thetis polymeric microfluidic pad, able to immobilize cells in a small and confined space was utilized in cases with a proportion of PC <1% after PC positive selection by immunomagnetic bead separation with anti-CD138 Ab. Ig heavy chain (IGH) translocations t(11;14), t(4;14), t(14;16), 13q/14, 17p13 deletion and 1q21 gains were evaluated in 6 monoclonal gammopathy of uncertain significance (MGUS), 6 smoldering MM (SMM) and 3 multiple myeloma (MM).

Results: Despite the low number of BM PC, Thetis nanotechnology documented chromosomal abnormalities in 80% of cases: 3/6 MGUS, 5/6 SMM and 3/3 MM. The most common genetic alteration was chromosome 11 trisomy, present in 2 MGUS and 3 SMM, followed by chromosome 13 deletion identified in 3 SMM and 1 MM, t(11;14) was present in 1 SMM, hypodiploidy, an adverse prognostic alteration, was documented in 1 SMM and 1 MM, 1q gain in 1 MM.

Conclusions: A low proportion of tumor cells represents a major disadvantage in cytogenetic studies at diagnosis or for residual disease monitoring after treatment. The Tethis miniaturized FISH approach allowed the genetic study in samples with a low percentage of PC, with a significant reduction in probe usage and minimal cell requirements, suggesting a relevant role for molecular cytogenetic screening of PC malignancies with low percentage of BM infiltration.

PO031

LONGITUDINAL STUDY OF MUTATIONAL LANDSCAPE OF MYELOMA CELLS ACROSS TREATMENT WITH LENALIDOMIDE

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Introduction: The potential effect of anti-myeloma drugs on clonal

selection is currently under investigation. Continuous Lenalidomide (Len) treatment is becoming the backbone of MM therapy, however the potential role of Len in the clonal selection and the emerging of resistant clones are unknown.

Methods: Firstly, we analyzed the sub-clonal evolution in two human myeloma cell lines (HMCLs, RPMI-8226 and JJN3) after long-term treatment with Len (2 μM), to mimic the continuous treatment regimens of multiple myeloma (MM) patients, by CGH+SNP arrays and FISH tests (Probes: IGH b.a., c-MYC b.a.), collecting both HMCLs and vehicle (DMSO) samples at starting point (T0), 1 mth and 2 mths of treatment.

Then we confirmed and expanded data, performing FISH with a wide panel probes [IGH b.a., CCND1-MYEOV/IGH, FGFR3 /IGH, MAF/IGH, 17p13+ATM, 1q21/1p32, 13q14 +CEN12, D5S721/D5S23, CEP9, CEP15] on primary purified CD138+ cells of 9 MM patients (pts) before and after Len treatment (median time of Len treatment: 18 months; range 5-63 months) either continuously with Len in combination with dexamethasone and/or alkylating agents (5 pts) or sequentially with proteasome inhibitors (PI) followed by Len (4 pts). Two pts were treated with Len-based treatment upfront while 7 at relapse. An aberration was defined as being as subclonal if it was found in 10-60% of cells or as the major clone if it was found in more than 60%, with a cut off of 10% for all probes (Table 1).

Results: In all HMCLs, both copy number alterations (CNAs) and loss of heterozygosity (LOH) regions have been detected. Mainly, these alterations were not specific for Len treatment, being also detected in DMSO treated samples. FISH hybridization patterns of IGH b.a. and cMYC b.a revealed that the clonal composition reported at T0 did not change after Len treatment in the analyzed HMCLs. Regarding primary PCs, longitudinal iFISH studies revealed MM distinct evolutionary paths of disease between the two time points considered: in the group of pts treated with continuously Len, the treatment induced no clonal evolution in 2 out of 5 pts and a branching or linear evolution in the remaining 3 pts. On the other hand, all pts treated with PI followed by Len, showed a branching or linear evolution after Len treatment, with an expansion of the del(p53) best-fitting clone or acquired clonal heterogeneity at 1q21 level, with subclones harboring different degrees of amplification.

Conclusions: In conclusion, our analyses suggest that *in vitro* continuously treatment with Len was not associated with clonal evolution. On the other hand, a branching or linear evolution may occur *in vivo* in MM pts treated with different Len-based regimens mainly in a sequential manner. Our findings underline the possible role of iFISH, as single cell analysis, to disclose intra-clonal eterogeneity in MM pts and the need of larger prospective studies to reveal MM distinct evolutionary paths over a patient's disease course.

Table 1.

Patients	Time T0→T1 (months)	Clonal eterogenity	Deletion chr13	Deletion p53	IGH Aberrations	Hyperdiploydy	Amplification 1q	Deletion 1p	Treatment
MM1 T0	6	YES	NO	NO	NO	YES (100%)	NO	NO	Rd (6)
MM1R T1		YES	NO	NO	NO	YES (100%)	NO	NO	
MM2 T0	14	YES	YES (88%)	YES (65%)	NO	YES (100%)	YES (94%)	NO	Cy-Rd (8), Elo-Rd (2)
MM2R T1		YES	YES (78%)	YES (85%)	NO	YES (100%)	YES (89%)	NO	
MM3T0	18	NO	NO	NO	NO	YES (100%)	YES (5%)	NO	MPR (9); Rd (9)
MM3R T1		NO	NO	NO	NO	YES (100%)	YES (100%)	NO	
MM4 T0	63	NO	NO	YES (100%)	NO	YES (100%)	/	/	MPR (9); Rd (44)
MM4R T1		NO	YES (60%)	YES (92%)	NO	YES (100%)	NO	NO	
MM5 T0	9	NO	YES (89%)	YES (70%)	del	NO	YES (80%)	YES (15%)	MPR (3); Rd (5)
MM5R T1		NO	YES (100%)	YES (88%)	del	NO	/	/	
MM6 T0	25	YES	NO	YES (13%)	t(11;14) (100%)	NO	NO	NO	BBD (2); Cy-Rd (11); Rd (12)
MM6R T1		YES	NO	YES (16%)	t(11;14) (73%)	NO	NO	NO	
MM7 T0	24	NO	YES (100%)	YES (91%)	gain	NO	NO	NO	VTd (3); Rd (14); Cy-Rd (4)
MM7R T1		YES	YES (70%)	YES (79%)	gain	NO	NO	NO	
MM8 T0	5	YES	YES (90%)	YES (7%)	(t11;14) (100%)	NO	YES (100%)	NO	KRd (5)
MM8R T1		YES	YES (4%)	YES (78%)	(t11;14) (100%)	NO	YES (100%)	NO	
MM9 T0	34	NO	YES (70%)	NO	NO	YES	NO	NO	MPV (9); Cy-Rd (15)
MM9R T1		YES	YES (5%)	YES (96%)	NO	YES	YES (47%)	YES (93%)	

Legend: Rd= Len-Dexamethasone; KRd= Carfilzomib-Len-Dexamethasone; BBD=Bortezomib-Bendamustine-Dexamethasone; Cy-Rd Cyclophosphamide-Len-Dexamethasone; Rd-Elo= Len-Dexamethasone-Elotuzumab; MPR= Melphalan-Prednisone-Len; MPV= Melphalan-Prednisone-Bortezomib; VTd= Bortezomib-Thalidomide-Dexamethasone.

PO032

DARATUMUMAB MONOTHERAPY IN REAL LIFE COMMUNITY SETTING OF REFRACTORY MYELOMA PATIENTS: COMFORTABLE MANAGEMENT OF INFUSION-RELATED REACTIONS AT THE FIRST INFUSION

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Daratumumab (DARA) is a first in class CD38-directed monoclonal antibody approved for the treatment of relapsed and/or refractory multiple myeloma (RRMM). High occurrence of all grade of Infusion-Related Reactions -IRRs- has been described, inducing infusion delayed or interruption and need of patient hospitalization.

Starting from April 2017, 13 RRMM patients received DARA intravenously (i.v.) in monotherapy at dosage 16 mg/kg actual body weight weekly for the first 8 weeks and then every 2 weeks, accordingly to recommended schedule. The total dose was diluted in 1000 mL 0.9% Sodium Chloride. To prevent or reduce IRRs, the first dose was split in 2 administrations within 24 hours. One hour after standard premedication (including 60 mg methylprednisolone I.V., 1000 mg acetaminophen per os and 50 mg diphenhydramine I.V.) the first 250 ml were given starting at infusion rate of 50 mL/hour with a progressive increase at 100 mL/hour. After 18 hours, premedication was given and the remaining 750 mL were infused at a rate of 100 ml/hour with a progressive increase at 200 mL/hour. Thus, we evaluated 13 heavily pre-treated patients (n=6/7 M/F, median age 61 years, range 46-74), most of them double refractory (n=11, median of 5 previous lines, range 1-13). In 4/13 patients there was high risk of IRRs due to a clinical history of known obstructive pulmonary disease and recurrent episodes of pneumonia. At the first day of infusion, 10/13 patients had a grade 1-2 IRR, started when the infusion rate was 100 mL/hour and treated with suspension of infusion for about 20-30 minutes followed by infusion of antipyretics or antihistamine. Restarting DARA infusion at 50 ml/h was safe and comfortable. IRRs were cough in 4 patients, congestion and runny nose in 4 patients, throat tightness and dyspnea in 3 patients and chills in 1 patient. Rarely, a second manifestation of IRRs occurred again when infusion rate was incremented at 100 or 150 ml/h. Symptoms were treated in the same way and resolved promptly. None of our patients discontinued treatment for IRR. Treatment was safe and well-tolerated: 6/13 patients had severe adverse events (anemia and neutropenia ≥ grade 3) and 2/13 pneumonia grade 3, requiring hospitalization. Despite complete remission has not yet been achieved, clinical benefit was evident for 8/13 patients, with 46% of overall response rate (after 8 infusions). Since expression of CD38 on MM cells correlates with DARA activity *in vitro*, we monitored the mean intensity fluorescence (MFI) of CD38 in peripheral blood circulating plasma cells (cPC) by flow cytometry. At baseline, cPCs were detectable in 8/12 patients, CD38 MFI was higher in patients with extra-medullary localization (p=0.02), but no changes could be appreciated after 2 cycles of DARA. In conclusion, DARA monotherapy is efficacious and safe in RR/MM. IRRs are weakly and occur only the first day, and they can be reduced by splitting the infusion over 24 hours.

PO033

LOW IGM CONCENTRATION PREDICTS TIME TO PROGRESSION OF MGUS TO MULTIPLE MYELOMA: RESULTS FROM A RETROSPECTIVE SINGLE CENTER STUDY

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Background: Multiple Myeloma (MM) is a biologically and clinically heterogeneous disease, anticipated in virtually all patients by an asymptomatic phase with very low percentage of neoplastic plasma cells, known as monoclonal gammpopathy of undetermined significance (MGUS). Malignant transformation of MGUS through MM occurred in 7 to 19% of the patients at 5 to 10 years. Unfortunately, at this time, there is no single pathologic or molecular feature that can reliably identify patients with MGUS or smoldering myeloma (SM) with a high risk of evolution in MM. Indeed, a recent report (Kyle, 2018) showed that non-IgM MGUS have lower risk of progression than IgM MGUS, based on the presence of two adverse risk factors — namely, an abnormal serum free light-chain ratio (ratio of kappa to lambda free light chains) and a high serum monoclonal protein (M protein) level (≥1.5 g per deciliter).

Several recent reports identified immunoparesis, defined as reduction of not-involved immunoglobulins, as a predictor of progression to MM, but their role in the progression form MGUS through MM has not been extensively investigated .

Aims: Measuring the prognostic meaning of immunoparesis in progression from non-IGM MGUS to MM in a retrospective single center study.

Methods: We retrospectively examined the case records of 1300 non-IgM-MGUS patients who accessed to our center from January 2007 through November 2017, in order to identify those patients that progressed to multiple myeloma (N=50). In accordance with our clinical practice, bone marrow examination was deemed unnecessary unless the patient had unexplained anemia, renal insufficiency, or bone pain, and clinical follow-up. The following factors on the risk of progression have been evaluated: age, sex, hemoglobin, creatinine, albumin, the level and type of the serum and urinary M protein, and concentration of uninvolved immunoglobulins The cut off point for low concentration of IgM was based on the lower limit of the normal range (60 mg/dL). Time to progression(TTP) was calculated as the time between diagnosis of MGUS and the progression to SM or symptomatic MM according to 2017 IMWG criteria.

Results: Median age of patients at diagnosis of MGUS was 62 years (range 35-76), 21/50 of progressed MM patients were female. None of the above mentioned parameters was predictor of TTP with the exception of IgM levels. Median value of IgM at diagnosis of MGUS was 49 mg/dL. Indeed, only IgM<60 mg/dL at diagnosis of non-IgM-MGUS could identify patients with shorter time to progression to MM (2.75 versus 6.25 years, p=0.0024, Log-rank test).

Conclusions: Concentration of IgM<60 mg/dL at diagnosis of non-IgM-MGUS predicts time to progression to SMM or MM, providing a fast and cheap means for monitoring MGUS patients in clinical practice.

PO034

DEVELOPMENT OF CHRONIC MYELOGENOUS LEUKEMIA IN A PATIENT WITH MULTIPLE MYELOMA

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Introduction: Multiple myeloma (MM) is a hematologic malignancy caused by the proliferation of clonal plasma cells in the bone marrow, leading to uncontrolled production of monoclonal immunoglobulin. Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm associated with the presence of the BCR-ABL1 fusion gene. Therefore, the respective malignant cells in MM and CML are completely different, being lymphoplasmacytic and myeloid cells, accordingly. The occurrence of MM and CML in the same patient, either at diagnosis or with one or the other disease pre-existing,is a rare event that has previously been reported in a limited number of case reports in the literature. Here we reported a case of 65 years old male with Multiple Myeloma, diagnosed in 2014, who developed CML after three years from diagnosis.

Methods: Laboratory examinations including peripheral blood smears, bone marrow(BM) aspirate, blast percentage and BM cellularity. Flow cytometry immunophenotype analysis was performed using panel of antibodies designed for MM. Cytogenetics analysis was performed on BM aspirates according to standard laboratory protocol. A total of twenty G-banded methaphases were evaluated. LSI IGH dual color break Apart, LSI D13S319/13q34 and LSI TP53(17p13.1) probes (Abbott molecular) were used to performed FISH analysis.

Results: At diagnosis of MM, BM biopsy revelead 80% monoclonal

plasma cell infiltration and flow cytometry demonstrated plasma cells positive for CD38,CD138,CD19 and negative for CD56 and CD45.After therapy with velcade, the patient underwent autologous transplantation of peripheral blood hematopoietic stem cells, obtaining complete remission of the disease.In December 2017 his peripheral blood exhibited progressive leukocytosis with a white cell count of 60.16x103 /µl, a hematocrit of (42.4%), a hemoglobin of(13.9g/dl) and platelet count was 145x103/µl. BM biopsy revelead a predominance of mature and intermediate forms of granulocytes. Chromosome analysis revealed 46,XY,t(9;22)(q34;q11).Treatment was initiated with Dasatinib at standard dose.In April 2018 at follow-up, bone marrow biopsy showed relapse of MM. FISH analysis was found to be negative for TP53(17p13.1) and LSI D13S319/13q34 while it revealed IGH rearrangement in 50% of cells.

Conclusions: The coesistence of MM and CML suggests either a different clonal evaluation from a common pluripotent malignant stem cell, or exposure to previous chemotherapy and radiation, or existence of a pro-carcinogenic environment, or a coincidence, or every each one of the above possibile factors. Further investigation of associated possibile causes is need to get to definitive conclusions.

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PO035

IMPORTANCE OF CUMULATIVE DOSE OF CARFILZOMIB IN COMBINATION WITH LENALIDOMIDE AND DESAMETHASONE: A REAL LIFE SINGLE CENTER RETROSPECTIVE STUDY IN RELAPSED REFRACTORY MYELOMA PATIENTS

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Background: The combination of the second-generation proteasome inhibitor (PI) carfilzomib, lenalidomide and dexamethasone (KRd) is promising in relapsed/refractory multiple myeloma (RRMM). Since no data are currently available about the effect of cumulative dose in RRMM patients receiving KRd, we investigated the impact of cumulative dose of Carfilzomib in RRMM.

Methods: From November 2016 to February 2018, 36 consecutive RRMM patients (median of previous lines 4, range 1-10), received KRd regimen, consisting in Carfilzomib 20 mg/m2 IV on days 1 and 2 of the first cycle, then 27 mg/m2on days 8, 9, 15 16 of the first cycle and days 1, 2, 8, 9, 15 and 16 of the subsequent cycles, Dexamethasone 20 mg was administered on days 1, 2, 8, 9, 15, 16 and lenalidomide 25 mg daily given orally on days 1–21 of each 28-day cycle.

Results: Patients had a median age of 62.5 years (range 49-77), half of them were classified as high risk patients, according to R-ISS, 55% were refractory to previous treatment and 45% relapsed.

At least 5 KRd cycles were given to 28 (77%) patients. Rate of > very good PR was 41% (15 patients), including 9 complete responses (CR), 6 (16%) partial remissions (PR), 11 (31%) minimal responses (MR). 1 patient maintained stable disease and 3 patients were primary refractory and died within first three cycles. Progression occurred in one-third of patients (12/36) who achieved less than VGPR within first four cycles. The percentage of CR increased along with time whilst the percentage of PD tends to stabilize and occurs mostly in heavily pre-treated patients.

Discontinuations and carfilzomib dose reductions due to adverse events were 10%, mainly due to severe hematological adverse events (febrile neutropenia and anemia). Median cumulative carfizomib dose at 2, 3, 4 and six cycles was respectively 480 mg (282 mg/m^2), 735 mg (435 mg/m^2) , 995 mg (589 mg/m^2) and 1522mg (890 mg/m^2).

PFS at 12 months was 67.3%. PFS at two months was significantly longer in patients who received the higher total dose group (>480 mg/m^2) (not reached vs 11 months, hazard ratio (HR) 0.18, p=0.01). The same results were obtained when we analyzed the two groups by dose per square meter (> or < 282 mg/m^2): (median survival 11 months vs. Not reached, HR 0.27, =0.03). Similarly, we found that PFS was significantly longer in the higher total dose group at three, four and six months with p values respectively of 0.02 ; 0.05. and 0.02.

17 patients previously treated with lenalidomide (group A), compared to 19 not exposed to lenalidomide (group B), showed lower PFS at 12 months (58.3% versus 86.1% , p=0.06).

Conclusion: Depth of response was time and dose dependent with an acceptable safety profile in RRMM patients treated with KRd. Continuing therapy in responding patients and/or proactive adverse events management, might influence efficacy. Patients not previously exposed to lenalidomide are the best candidate for a favourable outcome with KRd regimen.

PO036

CARFILZOMIB-LENALIDOMIDE-DEXAMETHASONE IN THE MANAGEMENT OF LENALIDOMIDE-REFRACTORY MULTIPLE MYELOMA

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Carfilzomib is an epoxyketone proteasome inhibitor of second generation, proved to be effective and safe in relapsed and refractory Multiple Myeloma (rrMM), in combination with dexamethasone or lenalidomide and dexamethasone. In this retrospective observational trial, it has been evaluated efficacy and safety of carfilzomib, in combination with lenalidomide-dexamethasone (KRD) as salvage regimen in patients with rrMM, refractory to lenalidomide, whose prognosis is particularly severe. 31 patients (19 M/12 F), with rrMM, median age at diagnosis 64 years (r. 47-82), median age at start of treatment 68 years (r. 48-84) previously treated with several lines of treatments (median 3, r. 2-11), underwent to KRD regimen (ASPIRE trial schedule) for a median treatment cycles of 4 (r 1-14). ISS was equally distributed, and cytogenetic was evaluable in 8 patients, and in particular one del13q14 1q gain, one del13q14 and one t(11;14). All patients had previously been treated with bortezomib and IMIDs, and were refractory to this agents. 61% (19/31) of them had undergone at least to a single autologous SCT. According to IMWG criteria, after a median follow-up of 3 months (r.1-13), ORR was 67,7% (21/31: 5 CR, 9 VGPR, 7 PR) with 4 progressive diseases (PD) and 6 patients in stable disease (SD): this can be considered as an impressive result in this subset of rrMM patients, refractory to lenalidomide. In particular, for 2 patients, KRD was, after having achieved at least a PR, a bridge to second autologous SCT. Median time to response was 2 months (r.1-4), median OS from diagnosis was 57 months (r. 9-170), median OS from start of Carfilzomib was 6 months (r. 1-14). Carfilzomib was well tolerated, with grade 2 anemia in 35% (11/31) of patients, successfully managed by ESAs, without necessity of blood transfusions; 19% (6/31) grade 3-4 neutropenia (pegfilgrastim in primary prophylaxis was given, no ospedalization was required, no septic shocks were observed); 32% (10/31) grade 2, 19% (6/31) grade 3 and 9% (3/31) grade 4 thrombocytopenia, without hemorrhagic events and necessity of transfusions. Concerning severe extra-hematologic toxicity, it was observed pneumonia in 45% (14/31) of patients, treated by common antibiotic drugs; hypertension (grade 2-3) in 35% (11/31) of patients; arrhythmias in 9% (3/31) of patients; dyspnea in 16% (5/31) of patients; fatigue in 32% (10/31) of patients. All patients were carefully monitored by expert cardiologists of our deparment. Carfilzomib-Lenalidomide-Dexamethasone has shown significant efficacy in a particularly severe setting of patients, relapsed and refractory to all available therapeutic resources, also lenalidomide, and, in particular cases, it could be considered as a bridge to a second autologous or allogenic SCT.

PO037

BENDAMUSTINE-BORTEZOMIB-DEXAMETHASONE (BVD) IN HEAVILY PRETREATED MULTIPLE MYELOMA

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Bendamustine is a bifunctional alkylating agent, with low toxicity, proved to be effective in relapsed, refractory and in new diagnosed Multiple Myeloma (MM). It has been evaluated efficacy and tolerance of Bendamustine, in combination with bortezomib-dexamethasone (BVD) in patients with relapsed and refractory MM (rrMM), whose prognosis is particularly severe. A regional retrospective real-life analysis of patients with rrMM who had been treated with BVD as salvage therapy has been performed. 56 patients (31 M/25 F), with rrMM, median age at diagnosis 57.3 years (r. 36-82), median age at start of treatment 61.8 years (r.37-83) treated with several lines of treatments (median 6, r. 2-11), every refractory to all the drugs previously received (also Bortezomib), received BVD (Bendamustine 90 mg/sqm days 1,2; Bortezomib 1.3 mg/sqm days 1,4,8,11, Dexamethasone 20 mg days 1,2,4,5,8,9,11,12, Pegfilgrastim day +4) every 28 days, until progression. ISS was equally distributed, and cytogenetic was evaluable in 12 patients, and in particular one del13q and one t(11;14). All the patients had previously been treated with schedule containing bortezomib and IMIDs, and 30% had also received radiotherapy. 67% of them had undergone at least to a single auSCT. All patients were relapsed and refractory to last therapies received before BVD. Bendamustine was well tolerated, with grade 3 transfusion-dependent anemia in 41% of patients, and 37% grade 3 neutropenia (no ospedalization was required, no septic shocks were observed). No severe extrahematologic toxicity was observed, only grade 1 gastrointestinal side effect (nausea), treated by common antiemetic drugs. According to IMWG, after a median follow-up of 14 months (r.2-36), ORR was 64% (36/56: 4 CR, 7 VGPR, 16 PR, 9 MR) with 8 PD and 12 patients in SD, which can be considered as an impressive result in this subset of rrMM patients. In particular, for 11 patients, BVD was, after having achieved at least a PR, a bridge to second auSCT, and for two patients a bridge to alloSCT. Three patients have shown a notable PR after failure of novel agents (i.e. Carfilzomib and Pomalidomide). Median time to response was 1.2 months (r.1-3), median OS from diagnosis was 62.7 months (range 6-151), median OS from start of Bendamustine was 9.8 months (range 2-36). The triplet Bendamustine-Bortezomib-Dexamethasone has shown significant efficacy in a particularly severe setting of patients, relapsed and refractory to all available therapeutic resources, and, in particular cases, it could be considered as a bridge to a second autologous or allogenic SCT.

Benign Hematology (Red Cell Disease Thrombosis and Hemostasis)

PO038

ILIOPSOAS HAEMATOMA AND THROMBOCYTOPENIA DABIGATRAN INDUCED: A RARE COMPLICATION OF NOACS

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Introduction: Iliopsoas haematoma is a rare complication that occurs in patients receiving anticoagulant therapy. The clinical manifestation of iliopsoas haematoma is non-specific. It can mimic orthopaedic or neurological disorders, including paraesthesia or paresis of the thigh and leg due to compression of the nerve plexus. Among the many available diagnostic modalities, computed tomography is the most useful radiological method for diagnosis. The report described a case of iliopsoas haematoma due to NOACs (dabigatran) with thrombocytopenia paraesthesia in the right leg who was successfully treated by conservative approach.

Methods: A 83-year-old Caucasian male patient was admitted to the Internal Medicine Department to Calcinate Hospital, with complaint of lower quadrant abdominal pain and paresis in the right leg and showed hemorrhagic necrotic skin lesions on his neck and right hand. Previous medical history included a pulmonary embolism, atrial fibrillation, splanchnic venous thrombosis, which was being treated with dabigatran 220 mg/die for thromboprophylaxis. On admission, he was haemodynamically stable. Examination revealed thigh bruising surrounding the right groin and tenderness on the right side of the abdomen. He reported paraesthesia over the anterior thigh. On neurological examination, right crural monoparesis with pain was revealed. Laboratory test results were haemoglobin of 10.2 g/dL; leukocyte count of 13800 mm^3; platelet count 64000 mm^3; prothrombin time of 21.6 seconds (normal, 10 – 14 seconds). Enhanced CT of the abdomen showed the presence of a haematoma of the right iliacus and psoas muscles. An CT scan showed iliopsoas haematoma as an high density mass of 86,5 mm in the retroperitoneal space, with enlargement of the involved muscles.

Results: He was administered dabigatran (220 mg/day) for three days and his platelet count decreased abruptly (64000/μL). This suggested that dabigatran had caused thrombocytopenia and purpura; therefore, dabigatran administration was discontinued and was administered prednisone 25 mg/day for 14 days. The results of a blood test showed that the platelet count had recovered to the normal range of more than 150,000/μL. A conservative approach was performed for iliopsoas haematoma.

Conclusions: Dabigatran application may cause bleeding; therefore, careful monitoring during dabigatran treatment is required to prevent thrombocytopenia. Symptoms of iliopsoas haematoma are nonspecific, and may include muscle dysfunction and by the haematoma can lead to paraesthesia or paresis of the thigh and leg. Diagnosis is based on clinical manifestations and imaging studies such as ultrasonography and contrast-enhanced computed tomography (CT). A few studies, however, reported that dabigatran can cause thrombocytopenia, although the underlying mechanism remains unclear. Thus, an antidote for dabigatran was developed to prevent thrombocytopenia.

PO039

CHARACTERIZATION AND OUTCOMES OF ATTP PATIENTS WITH INITIAL OR RECURRENT DISEASE: EXPERIENCE FROM THE PHASE III HERCULES TRIAL OF CAPLACIZUMAB

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Introduction: Acquired thrombotic thrombocytopenic purpura (aTTP) is a life-threatening autoimmune blood clotting disorder characterized by severe thrombocytopenia, microangiopathic hemolytic anemia, and organ ischemia. Inhibitory autoantibodies cause a severe deficiency of the von Willebrand factor (vWF) cleaving enzyme ADAMTS13, leading to intravascular vWF-platelet aggregation and microvascular thrombosis. Patients are at risk for significant morbidity and death during each episode The mainstays of treatment are plasma exchange (PE) and immunosuppression. Caplacizumab, a bivalent Nanobody, targets the A1 domain of vWF, inhibiting the interaction between ultra-large vWF and platelets. Efficacy and safety data were obtained in the Phase III HERCULES study with caplacizumab in patients with aTTP (Scully et al., Blood 2017 130:LBA-1).

Table 1. Efficacy outcomes by previous TTP episode (Intention-to-treat population)

Efficacy outcomes	Initial Episode		Recurrent Episode	
	Caplacizumab N=48*	Placebo N=34	Caplacizumab N=24	Placebo N=39
Time to platelet count response Platelet count normalization rate ratio (95% CI)	1.67 (1.025 to 2.722)		1.64 (0.951 to 2.818)	
TTP related death, recurrence of TTP or a major TE event during the study drug treatment period	6 (12.8)	19 (55.9)	3 (12.5)	17 (43.6)
Recurrence of TTP during Overall Study period – n (%)	6 (12.8)	15 (44.1)	3 (12.5)	13 (33.3)
Refractory TTP – n (%)	0	1 (2.9)	0	2 (5.1)

* percentages are based on 47 patients (1 subject withdrew consent prior to study drug treatment)

Methods: In order to characterize disease presentation and evaluate treatment outcomes in patients enrolled in the HERCULES study with an initial or recurrent aTTP episode, demographics, baseline disease characteristics, and treatment outcomes (time to platelet count response, mortality, recurrence, major thromboembolic (TE) events and refractoriness) were evaluated for both subgroups using descriptive summaries.

Results: 145 patients were randomized, 82 with an initial aTTP episode and 63 with recurrent disease. Demographics were generally balanced between groups, whereas baseline disease characteristics were more severe in initial vs. recurrent episodes: mean platelet count ($28.8x10^9$/L vs. $44.4x10^9$/L), mean LDH (598U/L vs. 523U/L) and median cardiac Troponin-I (0.119µg/L vs. 0.036µg/L). The time from first symptoms until diagnosis was also longer in those experiencing an initial episode (6.5 days) vs. a recurrent one (3.9 days). More patients in the caplacizumab group had their first aTTP episode (66.7%) vs. the placebo group (46.6%). Treatment with caplacizumab improved outcomes [i.e., faster time to platelet count response, lower proportion of patients with either death, recurrence or a major TE event during the treatment period, lower recurrence rate during the overall study period, and prevention of refractoriness] in both subgroups compared to placebo (see Table 1 for detailed results).

Conclusions: Patients with an initial aTTP episode have a delayed presentation and more severe disease at baseline than those with recurrent disease. Treatment with caplacizumab improves outcomes in both subgroups.

PO040

TREATMENT OF ACQUIRED TTP WITH THE ANTI-VWF NANOBODY, CAPLACIZUMAB, RESULTS IN A SIGNIFICANT REDUCTION IN HEALTHCARE RESOURCE UTILIZATION - DATA FROM THE PHASE III HERCULES TRIAL

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Introduction: The efficacy and safety of caplacizumab, an anti-von Willebrand Factor (vWF) Nanobody, for the treatment of acquired thrombotic thrombocytopenic purpura (aTTP) were evaluated in the HERCULES study (Scully et al., Blood 2017 130:LBA-1).

Methods: The effect of treatment with caplacizumab on healthcare resource utilization was investigated. The volume and days of plasma exchange (PE), days in hospital and in the intensive care unit (ICU) were summarized and compared between the caplacizumab and placebo arms for the overall study drug treatment period, using a normal approximation to the Wilcoxon Rank Sum test.

Results: 145 patients were randomized, 73 to placebo and 72 to caplacizumab. Treatment with caplacizumab led to faster normalization of platelet counts, prevented exacerbations, and prevented patients from becoming refractory to treatment. This was reflected in a 38% reduction in mean (±SE) number of PE days in the caplacizumab (n=71) vs. placebo group (n=73): 5.8 (±0.51) days vs. 9.4 (±0.81) days ($p<0.001$). The mean (±SE) total volume of plasma exchanged was similarly reduced by 41%: 21.3 (±1.6) L vs. 35.9 (±4.2) L ($p<0.001$). Average duration of hospitalization (±SE) was decreased by 31% in the caplacizumab (n=71) vs. placebo group (n=73): 9.9 (±0.7) days vs. 14.4 (±0.7) days ($p=0.0025$). A third of the patients were admitted to the ICU (28 patients in the caplacizumab group, and 27 patients in the placebo group). In the caplacizumab group, the mean (±SE) number of days spent in the ICU was reduced by 65%: 3.4 (±0.4) days vs. 9.7 (±2.1) days ($p=0.0098$).

Conclusions: Caplacizumab, through rapid blocking of vWF-mediated platelet adhesion, represents a novel treatment for aTTP. Treatment with caplacizumab results in improved outcomes as reflected by meaningful reductions in healthcare resource utilization.

PO041

SAFETY PROFILE OF CAPLACIZUMAB DURING THE PHASE III HERCULES STUDY: FREQUENCY OF ADVERSE EVENTS ACCORDING TO STUDY PERIOD

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Introduction: Acquired thrombotic thrombocytopenic purpura (aTTP) is a life-threatening autoimmune blood clotting disorder. Patients are at risk for significant morbidity and death during each episode. Efficacy and safety data were obtained in the Phase III HERCULES study with caplacizumab in patients with aTTP (Scully *et al*., Blood 2017 130:LBA-1).

Methods: The frequency of treatment-emergent adverse events (TEAE) was analyzed, according to the following study periods: double-blind (DB) daily plasma exchange (PE) treatment period; DB post daily PE treatment period; and the treatment-free follow-up (FU) period. Analysis was performed on the study's safety population (i.e., all patients who received at least 1 administration of study drug).

Results: The safety population consisted of 71 caplacizumab-treated and 73 placebo-treated patients. The median (min; max) duration of DB study drug treatment was 35 (1; 65) days for the caplacizumab group and 23 (2; 66) days for the placebo group (i.e., shorter in the placebo group due to the switch to open-label caplacizumab treatment for those with a recurrence). During the overall study period, at least one TEAE was reported in 69 patients (97.2%) in the caplacizumab group and 71 patients (97.3%) in the placebo group. Of these, TEAEs were reported in 57/71 (80.3%) vs. 56/73 (76.7%) patients during the DB daily PE period, and in 52/65 (80.0%) vs. 57/64 (89.1%) patients during the DB post-daily PE period, in the caplacizumab vs. placebo group respectively. In the caplacizumab group, the most common reported TEAEs during the DB daily PE period were: epistaxis [9 patients (12.7%)], gingival bleeding [8 patients (11.3%)], and urticaria [11 patients (15.5%)], while during the post-daily PE period epistaxis [15 patients (23.1%)], headache [11 patients (16.9%)], and gingival bleeding [5 patients (7.7%)] were the most frequently reported TEAEs. In the placebo group, the most common TEAEs during the DB daily PE period were hypokalemia [11 patients (15.1%)], and insomnia, anxiety, chest pain, bruising, urticaria and pruritus (each in 5 patients (6.8%)], while during the DB post-daily PE period, TTP [28 patients(43.8%)], contusion [7 patients (10.9%)] and headache [5 patients (7.8%)] were the most frequently reported TEAEs. In the 28-day FU period, TEAEs were reported in 36/66 (54.5%) patients in the caplacizumab and 18/39 (46.2%) patients in the placebo group. The most commonly reported TEAEs in the caplacizumab group during the FU period were headache [5 patients (7.6%)] and TTP [6 patients (9.1%)]. During the FU period, all other TEAEs were reported in <5% of patients.

Conclusions: In line with its pharmacology, treatment with caplacizumab was associated with an increased risk of mucocutaneous bleeding. These events were reported with a similar frequency during the daily PE period (i.e., when platelet counts are low) and the post-daily PE period (i.e., when platelet counts are normalized).

PO042

VITAMIN D SUPPORT IMPROVES SUCROSOMIAL IRON EFFECTIVENESS IN VITAMIN D AND IRON DEFICIENT PATIENTS. MONOCENTRIC PROSPECTIVE RANDOMIZED STUDY

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Introduction: Vitamin d has an antiinflammatory effect inducing Th2 and Tregs response and m^2 macrophage polarization and hinibits hepcidin production.Aim of this study is to see if vitamin d support improves iron absorbtion in patient with sideropaenic anemia and vitamin d deficiency.

Patientsand and Methods: In group A 20 patients (M/F:1/2), median age 55 yo (R50-70),with vitamin d <10 ng/ml and with median Hb value 8.5 g/dl (R8-9.5), with median ferritin value 20 ng/ml (2-28)were supported with sucrosomial iron 30 mg tid for 3 months and cholecalciferol 4000 UI/day. In group A median CRP was 35 ng/ml (R20-47) and 6 patients had documented bacterial infection. In group B 20 patients (M/F:1/2), median age 60 yo (R55-68),with vitamin d <10ng/ml and with median Hb value 8.2 g/dl (R8-10), with median ferritin value 18 ng/ml (10-30) were supported with sucrosomial iron 30 mg tid for 3 months .In group B median CRP was 28 ng/ml (R20-32) and 7 patients had documented bacterial infection.

Results: In group A after 3 months patients achieved median Hb level of 12.5g/dl (R 10-13). Patients with bacterial infection achieved median Hb level of 10g/dl (R 9-11). In group B after 3 months patients achieved median Hb level of 11.5g/dl (R 9.5-12). Patients with bacterial infection achieved median Hb level of 9.5g/dl (R 9-10.5).

Conclusions: Vitamin D seems to improve sucrosomial iron effectiveness in patients with vitamin d deficiency, with the exception of patients with bacterial infection.

PO043

HLA DRB1*11:04 IN PATIENTS WITH IDIOPATHIC THROMBOTIC THROMBOCYTOPENIC PURPURA

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Introduction: The HLA system is located on the short arm of chromosome 6 and plays a decisive role in the genesis of the immune response. The class I MHC molecules are expressed on the membrane of all cells except the red cells, while the class II MHC molecules are expressed mainly on immunocompetent cells. Many of these molecules have been associated with the predisposition to the onset of autoimmune diseases. The presence of HLA-DRB1*11 is considered a risk factor for the development of acquired Thrombotic Thrombocytopenic Purpura (TTP) due to its ability to present the FINVAPHAR peptide (derived from the CUB2 domain of the ADAMTS molecule 13) to the dendritic cells.

Aim of the study: Verify the association between HLA-DRB1*11:04 and TTP, through high-resolution HLA genomic typing of patients with TTP.

Materials and methods: We evaluated the HLA system in seven patients with remission of TTP and without clinical and laboratory signs of the disease, comparing it with the register IBMDR of donors of Basilicata. The molecular study of the HLA phenotype was performed accord-

ing to the following methods: PCR-sequence-specific-oligonucleotide probe hybridization (SSOPH) and PCR-sequence-specific-primers (SSP).

Results: The DRB1*11 allele was present in all patients. The DRB1*11:04 was always present at high resolution typing and was always in association with DQA1*05:05. In 4 cases out of 7 the DRB1*11:04 was the only DRB1 allele. No DRB1*11:01 was present in patients.The data of our patients were compared with those obtained in the register IBMDR of donors of Basilicata. Among the 4657 potential tdonors, the DRB1*11 was present in 1059 cases (22.73%). Among the DRB1*11 positive, the DRB1*11:04 was present in 63.57% and was the only DRB1*11 allele in 15.9% of cases; therefore DRB1*11:04 was present in 14.44% of all donors. DrB1*11:01 was present in 45.3% of positive for DRB1*11 (10.29% of total donors).

Conclusions: Our data, still very limited, seem to confirm the correlation between allele DRB1*11 (in particular DRB1*11:04) and TTP, as reported in literature. TTP, although very aggressive and potentially fatal, is essentially an acute disease and has the possibility of very long remissions, even with immunosuppressive therapies of relatively limited duration. So, it seems interesting to investigate in the future the role of DRB1*11:04 (through its ability to present the antigenic fragments of ADAMTS13 to cells of the immune system), in determining the particular type of autoimmune response of TTP.

Myeloproliferative Disorders and Chronic Myeloid Leukemia

PO044

CALRETICULIN INS5 AND DEL52 MUTATIONS IMPAIR UNFOLDED PROTEIN AND OXIDATIVE STRESS RESPONSES IN HEMATOPOIETIC CELLS

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Introduction: Somatic mutations of calreticulin (CALR) have been described in approximately 30-40% of JAK2 and MPL unmutated Essential Thrombocythemia and Primary Myelofibrosis patients. CALR is a chaperone that localizes primarily in the endoplasmic reticulum (ER) where it is responsible for the control of proper protein folding and for calcium retention. Recent data have demonstrated that the TPO receptor (MPL) is essential for the development of CALR mutant-driven myeloproliferative neoplasms (MPNs). However, the precise mechanisms of action of CALR mutants haven't been fully unraveled.

Methods: In this study, in order to clarify whether CALR mutations may affect the functions of CALR in the ER under homeostatic conditions, CALR mutant variants were overexpressed in K562 cells.

Results: Our results showed that CALR mutants impair the ability to respond to the ER stress and reduce the activation of the pro-apoptotic pathway of the unfolded protein response, therefore allowing the accumulation of unfolded proteins and conferring resistance to ER-stress induced apoptosis. Moreover, our data demonstrated that CALR mutations induce increased sensitivity to oxidative stress, reducing the ability to counteract ROS intracellular accumulation and thus leading to increase oxidative DNA damage. Finally, we demonstrated that the downmodulation of OXR1 in CALR-mutated cells could be one of the molecular mechanisms responsible for the increased sensitivity to oxidative stress mediated by CALR mutations.

Conclusions: Altogether, our data identify a novel MPL-independent mechanism involved in the development of MPNs mediated by CALR mutants. CALR mutations negatively impact on: 1. the capability of cells to respond to oxidative stress leading to increase DNA damage and genomic instability; and 2. the ability to react to ER stress, causing resistance to UPR-induced apoptosis.

PO045

CIRCULATING MICRORNA PROFILE IN PRIMARY MYELOFIBROSIS

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Introduction: MicroRNAs (miRNAs) are small non-coding RNA molecules whose deregulation can contribute to the multi-step processes of tumorigenesis. Growing evidence suggests that circulating cell-free miRNAs are optimal biomarkers for an earlier cancer diagnosis and for predicting prognosis and response to therapy. As recently reported by our group, the miRNA expression profile of CD34+ cells from Primary Myelofibrosis (PMF) patients highlighted the contribution of several deregulated miRNAs (i.e. miR-155-5p, miR-34a-5p, miR-382-5p, and miR-494-3p) to the pathogenesis of PMF.

Methods: In order to study the circulating miRNA profile in PMF, we designed a Custom OpenArray plate containing 224 miRNA Advanced TaqMan assays to detect miRNAs that were already described to be relevant for several cancers and hematological malignancies. Hence, we analysed the profile of circulating miRNAs from 52 PMF patients and 20 healthy donors. All plasma samples were initially screened for RNA quality and we excluded haemolyzed samples through the detection of two miRNAs (hsa-miR-451a and hsa-miR-23a-3p).

Results: RT-PCR data analysis highlighted a different profile of circulating miRNAs in patients and normal donors, and identified 56 miRNAs that can discriminate PMF sample from healthy controls. In particular, PMF patients showed increased plasma levels of miR-34a-5p, miR-382-5p, and miR-494-3p according to their expression in PMF CD34+ cells.

Then, we investigated the correlation between miRNA plasma levels and clinical features of PMF patients. Interestingly, the levels of miR-494-3p and miR-99b-5p are upregulated, while miR-25-3p is decreased in the ""high molecular risk"" patients. Moreover, the increased level of miR-99b-5p in PMF plasma is correlated with low overall survival and high risk of leukemic transformation. Additionally, we found that miR-424-5p, miR-574-5p, and miR-92b-3p levels are positively correlated with the severity of bone marrow fibrosis. Finally, we found several miRNAs, which level is increased in plasma of PMF patients with thrombotic event (i.e. miR-151a-5p, miR-425-3p, miR-548-5p).

Conclusions: We described for the first time the circulating miRNA profile in PMF. In particular, we found that the levels of miRNAs in plasma of PMF patients differ significantly from controls. Our analysis revealed that circulating miR-99b-5p is elevated in PMF patients, and that this upregulation is associated with leukemic transformation and short overall survival. Our findings suggest that circulating miR-99b-5p might be a novel prognostic biomarker for PMF patients. Moreover, we reported miR-424-5p, miR-574-5p, and miR-92b-3p as fibrosis markers suggesting a putative pro-fibrotic role for these miRNAs in PMF patients.

PO046

RUXOLITINIB DECREASES NUMBER OF CIRCULATING ENDOTHELIAL CELLS AND CRP VALUE IN PATIENTS WITH SPLANCHNIC VEIN THROMBOSIS(SVT) AND PRIMARY MYELOFIBROSIS(PMF)TREATED WITH VITAMIN K ANTAGONISTS(VKA)

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Introduction: PMFoften causesSVT.Data regarding outcome of this subset of patients, mainly if treated withVKAplus ruxolitinib,are very few. Aim of this case series is to analyze if use of ruxolitinib+VKA is safe and effective in treatment of patients withPMF with IPSS INT-2.

Methods: This study is a retrospective study. 4female patients, median age47(R35-55), withPMF INT-2 and with SVT(2portal, 2mesenteric+splenic+portal), median Hb11.5g/dl(R11-12.5), PLT90000/mcl(R70000-100000), WBC10000/mcl(R4000-11000), peripheral blood blasts 1%(R1-2), 1 patient heterozygous for factor V Leiden received ruxolitinib20mg/day+warfarin. All patients were Jak-2 mutated(GROUP1).

In an historical cohort 6patients 2male,4female median age60(R45-65), 3PV,1ET,1PMF, 1 paroxysmal nocturnal hemoglobinuria and with SVT(4portal,2splenic+portal),median Hb12.5g/dl(R10-13.5), PLT1 50000/mcl(R110000-200000), WBC8000/mcl(R6000-10000), peripheral blood blasts 0%(R0-1), 1 patient heterozygous for factor V Leiden, 1 for prothrombin G20210, received Hydroxyurea 1000 mg(R500-1500)/day+warfarin. 3 patients were Jak-2 mutated (GROUP2).

In an other historical cohort 6patients 4male,2female median age60(R55-70), 3liver cirrhosis,3solid cancer and with SVT(5portal,1splenic+portal),median Hb11.5g/dl(R9-12.5), PLT 100000/mcl(R90000-130000), WBC4000/mcl(R2000-9000), peripheral blood blasts 0%,received only warfarin. 1 patient was Jak-2 mutated (GROUP3). All patients received an abdominal vascular doppler echography, C reactive protein(CRP) and D-Dimers(DD) mesuration at0, 3and6 months of follow-up. In patients receiving ruxolitinib circulating endothelial cells(CEC) were measured at same time (in flow cytometry with BDantibody CD146).

Results: Patients of GROUP1 showed a complete resolution of SVT in 2 cases and a partial portal recanalization in 2 cases after 3 months, without any thrombosis relapse or progression after 6 months; patients of GROUP2 showed a complete resolution of SVT in 2 cases, a partial portal recanalization in 2 cases and no resolution in 2 cases only after 6 months; patients of GROUP3 showed a partial portal recanalization in 3 cases, no resolution in 2 cases and 1 progression only after 6 months.

MedianCRP after0, 3and6 months(mg/l) was respectively inGROUP1 27(R18-33),8(R3-10),6(R3-7),inGROUP230(R20-35),18(R12-22), 10(R7-15), in GROUP3 38(R28-40), 35(R27-42),33(R22-35).

MedianDD after0, 3and6 months(ng/ml) were respectively inGROUP1 6000(R4500-7000), 500(R300-1000),130(R200-300), inGROUP2 4500(R3200-5500), 900(R850-2100),400(R350-800), in GROUP3 2800(R1300-3200),4700(R3700-4900),3500(R3000-4800).

MedianCEC/ml inGROUP1 after0, 3and6 months(mg/l) were respectively 1500(R800-5500),800(R600-1800),500(R400-1200).

No patient showed side effects treatment related.

Conclusions: Ruxolitinib and VKA is safe and effective and shows a faster recanalization and an antinflammatory effect in patients with PMF with SVT. These results needs confirmation on a larger cohort of patients.

PO047

TOWARD THE IDENTIFICATION OF A MICRORNA-BASED SIGNATURE OF CIRCULATING MICROPARTICLES FROM TRIPLE NEGATIVE AND JAK2(V617F) MUTATED PATIENTS WITH MYELOFIBROSIS

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Introduction: Myelofibrosis (MF) is characterized by clonal hemopoiesis, inflammatory microenvironment and mutations in JAK2, MPL, or CALR genes. Around 10% of patients (pts) do not carry the 3 mutations (Triple negative, TN). Microparticles (MPs; 0.1 - 1 μm) are small vesicles deriving from the cell plasma membrane with a role in intercellular signalling. Circulating MPs are increased in inflammation and cancer including MF. MicroRNAs (miRs) contribute to MF pathogenesis. However, the miRs profile of MPs has never been investigated in MF.

Here we studied miR expression of circulating MPs from JAK2(V617F) mutated and TN pts.

Methods: Peripheral blood was collected from 6 MF pts (at diagnosis or out of cytotoxic treatment for at least 3 months) and 3 age/sex-matched healthy donors (HD). MF pts were JAK2(V617F) mutated (n=3) and TN (n=3). MPs were purified from platelet poor plasma by ultracentrifugation and quantified using the Nanosight technology. MiR expression of isolated pts/HD-MPs (10^9) was investigated using miRNeasy Mini Kit and TaqMan™ Array Human MicroRNA A Cards (ThermoFisher). RT-PCR validation assays are under way.

Results: As compared with the HD counterparts, many miRs were significantly upregulated in MPs from both JAK2(V617F) mutated and TN pts. Among them miR-21, known to be the most commonly upregulated miR in haematological tumours, is an anti-apoptotic factor with oncogenic potential and able to inhibit megakaryocyto-erithropoiesis. MiR-155, 222, 24 were upregulated in TN-MPs only. MiR-155 is upregulated in response to inflammation and, targeting p53 pathway, inhibits apoptosis. Its overexpression stimulates granulo-monocytopoiesis while impairing megakaryocytopoiesis. MiR-221/222, markers of poor prognosis in aggressive tumours and both increased in TN-MPs, show anti-apoptotic effect acting downstream of the oncogenic RAS-RAF-MEK pathway. MiR-423-5p, a pro-apoptic miR, shows high expression in JAK2(V617F)/TN MPs but is almost absent in the HD counterparts. MiR-34a-5p, shown to be associated with MF, is upregulated in pts-MPs; however, its expression is lower in TN-MPs. Interestingly, many miRs regulating inflammation (miR-21, 146a, 223, 19a) and proliferation (miR-199a-3p, 21, 99b) were overexpressed in MPs-pts. Finally, miR-155, 146a, 19a and 194 overexpression affects the JAK-STAT pathway by blocking SOCS3, a negative regulator of the JAK-STAT signalling, and promoting cell survival. Comparing TN vs JAK2(V617F) MPs, the expression of 6 miRs (miR-122, 27a, 744, 584c, 365, 483-5p) was increased, whereas 2 onco-suppressors miRs (let-7b and miR-361) were less expressed. Of note, 6 out of the above mentioned 8 miRs have SRSF1 gene as a common target which is both a protooncogene and a splicing regulator.

Conclusions: Circulating TN-MPs show distinct miR signature as compared with the JAK2(V617F) mutated counterparts. This study has the potential to identify disease-related biomarker(s).

PO048

WT1 GENE EXPRESSION AS PREDICTIVE AND PROGNOSTIC MARKER IN CHRONIC MYELOID LEUKEMIA

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Introduction: In Chronic myeloid leukemia (CML), about one third of patients reach a suboptimal molecular response nevertheless the use of TKIs. Physicians need ab initio to estimate the probability of treatment failure; for this purpose, they use several risk scores systems, such as Sokal. Nevertheless, the predictive power never reaches 100%, and the identification of alternative predictive factors is today object of great interest. Wilms' tumor 1 (WT1) gene is located on the chromosome 11 and acts as oncogene. At diagnosis it is over-expressed in several hematological malignancies, including 80% of Acute Myeloid Leukemias (AML). Few data concern CML.

Aim of the study: We want to retrospectively investigate the WT1 expression in 58 CML patients and to assess its eventual ability of predicting treatment failure.

Patients and Methods: WT1 and BCR/ABL quantitative PCR assays were performed at diagnosis, and after 3, 6, and 12 months of therapy on the RNAs extracted by peripheral blood samples of 58 CML patients in chronic phase (33 male and 25 female, median age 58 years).

Results: At diagnosis, WT1 levels were not significantly different according to age, sex or to the baseline Sokal score, but we observed that WT1 levels show high inter-patients variability, (median 61.8±307, range 0-1805). During follow-up, an evident reduction of WT1 expression was observed. 15/58 cases presented a failure of therapy during follow up (defined as BCR-ABL1 >10% at 6 months of treatment, >1% at 12 months, or confirmed loss of MR3 anytime) and WT1 expression levels at diagnosis were able to predict the occurrence of TKI change So, in order to assess if its levels could condition the long-term EFS, a ROC analysis, based on WT1 levels at diagnosis was performed; with a sensibility of 69% and a specificity of 76%, we defined a cut-off value of 156 (WT1/ABL1*10-4) as value useful to discriminate patients with good or bad long-term outcome. According to this value, EFS resulted significantly longer for cases with low WT1 levels (85%) in respect of the high-WT1 subgroup (44%) (p=0.001) and WT1 expression values were also able to discriminate the outcome inside different Sokal subgroups (Figure 1). The WT1 negative prognostic power could be overcome by the type of TKI adopted (imatinib vs second-generation TKIs): in low WT1 cases the choice of a second-generation TKI as first-line treatment did not change the EFS length (3y-EFS: 82% for cases receiving imatinib vs 92% for those treated with nilotinib or dasatinib). In the high-WT1 cohort, the use of nilotinib or dasatinib significantly improved the 3-years EFS (61% with second-generation TKIs vs 30% with imatinib).

Conclusions: Our study suggests that WT1 could be considered an useful predictive and prognostic marker in CML, and that performing a simple RQ-PCR for its quantitation could help physicians to better plan ab initio the more correct treatment and follow-up.

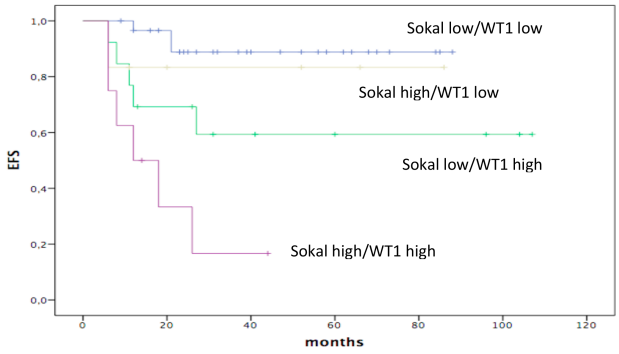

Figure 1. EFS related to WT1 levels and sokal risk.

PO049

ABCG2 AND ABCB1 RNA LEVELS AT DIAGNOSIS AND DURING THERAPY WITH IMATINIB AND 2G-TKIS IN NEWLY DIAGNOSED CP-CML PATIENTS

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Introduction: Overexpression of multidrug resistance proteins ABCB1 and ABCG2 confers resistance to anticancer drugs, including tyrosine kinase inhibitors (TKIs). However, little is known about the significance of ABCB1 and ABCG2 expression during TKI treatment for chronic myeloid leukemia (CML) and possible correlation with response to therapy. We aimed to investigate the role of ABCB1 and ABCG2 expression during the first 12 months of treatment with imatinib and 2G-TKIs for chronic phase CML patients and correlation with molecular response.

Methods: We analysed expression of ABCB1 and ABCG2 RNA at diagnosis and after 3, 6 and 12 months of TKI therapy in 49 CML patients receiving therapy with imatinib (n=32), nilotinib (n=10) or dasa-

tinib (n=5); patients were divided according to response (optimal vs warning/failure) at 3 months (optimal = BCR/ABL <10%), 6 months (BCR/ABL <1%) and at 12 months (BCR/ABL <0.1%). Patients that switched to alternative treatment after at least the 3rd month were included among non-optimal patients at the time of switch. RNA levels were expressed using the 2-ΔΔCt method proposed by Livak and Schmittgen, using ABL as normalizing gene and the expression level of the K562 cell line as calibrator, to standardize the Results:

Results: Mean ABCB1 and ABCG2 expression at diagnosis were 0.19±0.23 and 2.49±3.27, respectively. ABCB1 RNA levels increased during treatment, without differences among responding and non-responding patients at 3 (0.86±0.34 vs 0.99±0.82; p=0.64), 6 (0.95±0.31 vs 0.82±0.45; p=0.44) and 12 (1.16±0.62 vs 0.82±0.37; p=0.13) months. Similarly, ABCG2 expression increased in both groups during treatment: RNA level was 7.58±11.00 vs 39.66±80.03 (p=0.22) in optimal and non-optimal patients at 3 months, 7.01±6.05 vs 8.78±9.16 (p=0.61) at 6 months and 8.13±6.22 vs 9.34±9.10 (p=0.73) at 12 months. Considering RNA levels at diagnosis and early response to therapy (i.e. molecular response at 3 months), ABCB1 was expressed at almost identical baseline levels in the 34 patients with BCR/ABL <10% (0.19±0.24) and in the 13 patients without optimal response (0.22±0.22) (p=0.77). Conversely, ABCG2 RNA level at diagnosis was significantly lower in responding patients (1.68±1.56) compared to those with 3-month BCR/ABL ≥10% (4.61±5.53) (p=0.005); this difference was confirmed both in patients receiving imatinib (1.57±1.34 vs 4.31±5.95; p=0.04) and in those treated with 2G-TKIs (1.93±2.05 vs 5.10±4.77; p=0.09).

Conclusions: With the limits of a relatively small number of patients, our data suggest that high ABCG2 levels at diagnosis seems to be associated with non-optimal response to imatinib and 2G-TKIs. Kinetics of ABCB1 and ABCG2 RNA increase during treatment and possible correlations with response warrant further investigations.

PO050

CHARACTERIZATION OF THE SIGNALING PATHWAYS DRIVING LEUKEMIC EVOLUTION OF MYELOPROLIFERATIVE NEOPLASMS

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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. Evolution to acute myeloid leukemia (AML) occurs in 15-20% of PMF and 10% of PV/ET and is unresponsive to conventional therapy. PMF arises as a consequence of the acquisition of multiple somatic mutations in a single hematopoietic stem cell. The order of acquisition of these mutations could allow us to distinguish passenger mutations from possible disease-initiating events. Recent studies have shed light on the somatic mutations that contribute to transformation to AML, but the molecular mechanisms underlying progression to leukemia are not yet defined.

Methods: As a first step to unravel the molecular mechanisms associated with evolution to acute leukemia we performed RNA sequencing in 15 MPN patients who progressed to leukemia (paired samples). Among differentially expressed genes (DEGs), we focused our attention on transcripts involved in oxidative stress response. In order to unveil the role played by these genes during the leukemic progression, gene silencing experiments in primary human CD34+ cells have been performed.

Results: As a whole, our analysis identified 213 differentially expressed genes (DEGs) in the comparison AML vs PMF in paired patient samples. Gene Set Enrichment Analysis on DEGs showed several signaling pathways differentially expressed during leukemic transformation: TNFα signaling via NFKB, Hypoxia, Cellular response to oxidative stress, P53 pathway and KRAS signaling. Of particular interest in the PMF field is the deregulation of genes involved in oxidative stress response: previous studies reported that JAK2V671F induces the accumulation of reactive oxygen species (ROS) in a knock-in mouse model of MPN and in JAK2-positive MPN pts. Among DEGs in the comparison chronic vs acute phase, Hexokinase 3 (HK3) is strongly downregulated: HK3 has been described to exerts protective effects against oxidative stress, by increasing ATP levels, reducing oxidant-induced ROS production and preserving mitochondrial membrane potential. In line with these findings, our preliminary results show that HK3 silencing in Hematopoietic Stem Progenitor Cells (HSPCs) reduces the ability of cells to counteract the intracellular ROS accumulation and the DNA damage induced by oxidative stress.

Conclusions: As a whole, our analysis identified a set of DEGs in the comparison AML vs PMF paired patient samples. Among those, HK3 negatively impact on the capability of cells to respond to ROS accumulation and to oxidative stress-induced DNA damage.

PO051

CIRCULATING CD34+ STEM/PROGENITOR CELLS FROM TRIPLE NEGATIVE PATIENTS WITH MYELOFIBROSIS SHOW DIFFERENT NUMBER, GENE EXPRESSION PROFILE AND *IN VITRO* RESPONSE TO INFLAMMATORY STIMULI AS COMPARED WITH THE JAK2(V617F) MUTATED COUNTERPARTS

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Introduction: Myelofibrosis (MF) is characterized by clonal hemopoiesis, inflammatory microenvironment and mutations in JAK2, MPL, or CALR genes. Around 10% of patients (pts) does not carry the 3 driver mutations (Triple negative (TN)) displaying, along with lower haemoglobin level and platelet number, significantly worse survival-rates. The malignant hemopoietic stem/progenitor cells compartment of TN MF pts has never been characterized. Here, we compared the *in vitro* effects of crucial factors of the inflammatory microenvironment on the functional behaviour of TN circulating CD34+ cells vs the JAK2(V617F) mutated counterparts.

Methods: Peripheral blood was collected from 9 MF pts (at diagnosis or out of cytotoxic treatment for at least 3 months) and 10 age/sex-matched healthy donors (HD). MF pts were JAK2(V617F) (n=5) mutated and TN (n=4). Circulating CD34+ cells from pts were enumerated by flow cytometry. Immunomagnetically isolated CD34+ cells were *in vitro* treated with selected inflammatory factors (Interleukin (IL)-1β, Tumor Necrosis Factor (TNF)-α, Interleukin-6) for 12/24 hours and migration/survival ability was investigated. The plasma concentration of these cytokines was evaluated by ELISA. Gene expression profiling analysis (GEP) was performed in CD34+ cells from 3 TN vs 3 JAK2(V617F) mutated pts (Human Transcriptome array 2.0, Affymetrix) and gene set enrichment analysis was conducted by GSEA.

Results: GEP analysis showed a total of 165 genes differentially expressed (121 downmodulated and 44 upregulated in CD34+ cells from TN- vs JAK2(V617F) mutated pts). Specifically, we found that the expression of selected anti-apoptotic (TSPYL5, GFI-1 and FCMR) and pro-apototic (TNFSF10, TP53INP1) genes was significantly down- and up-regulated, respectively, in TN-CD34+ cells. Consistent with the GEP data, the *in vitro* survival of untreated TN-CD34+ cells was significantly decreased as compared with the JAK2(V617F) mutated counterparts. GEP analysis identified also a network of genes involved in cell adhesion, proliferation and inflammation which were mainly down-regulated in

TN-CD34+ cells. Specifically, GSEA highlighted an enrichment of cell adhesion, migration and chemotaxis signatures in TN-CD34+ cells. Accordingly, we found that the absolute number of circulating CD34+ and CD34+ CD184+ cells was significantly increased in TN pts as compared with the JAK2(V617F) mutated counterparts ($p<0.05$). Plasma concentration of IL-1β, IL-6 and TNF-α was significantly increased ($p<0.05$) in MF pts with no differences between the two groups. However, at variance with the CD34+ cells from the JAK2(V617F) mutated pts, the survival and migration of TN-CD34+ cells was not significantly affected by the combined IL-1β, IL-6 and TNF-α.

Conclusions: Altogether these findings suggest that TN-CD34+ cells show distinct *in vitro* functional features which may contribute to explain, at least in part, the defective hemopoiesis of TN pts.

PO052

POLO-LIKE KINASE-1, AURORA KINASE A AND WEE1: NEW THERAPEUTIC TARGETS IN SYSTEMIC MASTOCYTOSIS

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Introduction: Systemic mastocytosis (SM) is due to the pathologic accumulation of neoplastic mast cells in one or more extracutaneous organ(s). Although midostaurin, a multikinase inhibitor active against both wild type and D816V-mutated KIT, improves organ damage and symptoms, most patients (pts) relapse or have resistant disease. It is well known that Aurora-A (AKA) over-expression promotes tumorigenesis, but its role in the development of cancer has not been fully investigated. Evidences from literature suggest that AKA may confer cancer cell chemo-resistance, inhibits p53, but enhances Plk1, CDC25, CDK1, and cyclin B1 to promote cell cycle progression. Other studies indicate that AKA suppresses RAD51, poly(ADP ribose) polymerase (PARP), and gamma-H2AX to dysregulate DNA damage response. In this study we aimed to investigate the role of AKA and Plk 1 in genomic instability and aggressiveness of advanced SM.

Methods: Experiments were conducted in the HMC-1 MC leukemia cell line and in primary neoplastic MCs obtained from 7 SM pts (indolent SM, n=3; aggressive SM, n=3 and MC leukemia, n=1). Protein expression and activation was assessed by Western Blotting. Apoptotic cell death and cell cycle distribution were evaluated by flow cytometry after annexin V and propidium iodide staining, respectively. Drug cytotoxicity was evaluated by clonogenic assays.

Results: HMC-1 cells as well as primary neoplastic MCs displayed hyper-phosphorylated AKA and Plk1. Danusertib (AKA inhibitor) and Volasertib (Plk1 inhibitor) inhibited growth and induced apoptotic cell death in HMC-1.1 (IC50=649nM and 443nM, respectively) and -1.2 cells (IC50=892nM and 808nM, respectively). Their growth-inhibitory effects were associated with mitotic arrest and activation of apoptosis. Cell cycle arrest was associated with increased levels of phospho (p)-Chk1 and p-Chk2, p-cyclin B1, p-cdc2 and p-Wee1. Apoptosis was demonstrated by the detection of the cleaved forms of caspase-3, -8, -9, and PARP. Wee1 inhibition by AZD1775 (500 nM) after 24h treatment with Danusertib or Volasertib (100nM), when cells were arrested in G2 phase and Wee1 was overexpressed and hyper-activated, resulted in a significantly higher apoptosis than that obtained from concomitant treatment with Danusertib or Volasertib (100nM) + AZD1775 (500 nM) for 48h. Moreover, both drug combinations induced a significant increase of the DNA double-strand break marker γH2AX and RAD51, suggesting that Wee1 inhibition promotes mitosis and propagates genomic instability. Finally, Danusertib and Volasertib synergize with AZD1775 in inhibiting HMC-1 clonogenic potential and in inducing apoptotic cell death.

Conclusions: Plk1 and AKA, alone or together with Wee1, are attractive therapeutic targets in neoplastic MCs. Repurposing Plk1 or AKA±Wee1 inhibitors in advanced clinical development for other indications is a therapeutic strategy worth to be explored in an attempt to improve the outcome of patients with advanced SM.

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PO053

ATYPICAL BCR-ABL BREAKPOINTS DISPLAY SELECTIVE RESPONSIVENESS TO TYROSINE KINASE INHIBITORS

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Introduction: The BCR-ABL oncoprotein is the culprit of CML as it transforms the hematopoietic stem cell by altering its survival and proliferation properties. The efficacy of multiple Tyrosine Kinase Inhibitors (TKIs) on the most common BCR-ABL variants e1a2 (p190), e13a2 or e14a2 (p210) has been well established. However, several alternative breakpoints involving different BCR and/or ABL exons have been previously described but their TKI sensitivity has yet to be characterized. We analyzed 50 CML patients that did not present the canonical e1a2, e13a2 or e14a2 rearrangements and found three atypical BCR-ABL breakpoints in five subjects: one e12a2ins/del mRNA, three 13a3 and one e14a3 transcripts. These atypical isoforms (with the addition of two further deletion mutants lacking the BCR DC2 domain or the ABL SH3 domain) were investigated for their catalytic activity, transforming potential and TKI responsiveness.

Methods: Canonical BCR-ABL and the indicated variants were subjected to in-vitro kinase assays to evaluate their catalytic efficiency or were lentivirally expressed in Ba/F3 and Rat1Myc cell lines to assess their transforming ability and TKIs responsiveness. Ex vivo experiments were also carried out employing CD34+ progenitors expressing these atypical BCR-ABL transcripts.

Results: The e14a3 isoform showed catalytic efficiency comparable to conventional BCR-ABL and significantly superior to e12a2ins/del, e13a3, BCR-ABLΔDC2 and BCR-ABLΔSH3. However, after lentiviral transduction in Ba/F3 cells, we found that canonical BCR-ABL and e13a3 were most effective in inducing IL3-independent growth, while the remaining variants were less biologically proficient. No differences were observed in anchorage-independent growth assessed using the Rat1Myc cell line. Interestingly, when we evaluated the TKI sensitivity of these variants we found that, compared to canonical BCR-ABL, all atypical rearrangements were significantly less responsive to Imatinib and Dasatinib but not to Nilotinib. Similar data were obtained in LTC-IC assays using CD34-positive cells isolated from patients expressing the e14a3 variant. Finally, three individuals diagnosed with the above-mentioned variants that received Imatinib as first line treatment have failed the drug and are currently benefiting from Nilotinib therapy.

Conclusions: Our findings suggest that BCR and ABL regions flanking the BCR-ABL breakpoint play a critical role in leukemic transformation, selectively modulating TKI responsiveness. These findings have meaningful clinical consequences for CML patients displaying these atypical transcripts.

PO054

DIGITAL PCR IN PH+ CHRONIC MYELOID LEUKEMIA PATIENTS FOR RECOGNITION OF "STABLE" DEEP MOLECULAR RESPONSE AND IDENTIFICATION OF BEST CANDIDATES TO TKI DISCONTINUATION

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Introduction: Treatment of CML with TKIs aims to achieve a MMR and to possibly obtain a DMR, to gain the opportunity for TKIs discontinuation. Due to its intrinsic limitations qPCR cannot be considered as an optimal tool for DMR monitoring. The digital PCR (dPCR) has emerged as a more sensitive and accurate method to detect and monitor MRD. This study aims to comparatively evaluate qPCR vs dPCR for a better recognition of stable DMR and the best candidates to TFR.

Methods: 493 peripheral blood samples from 118 CML patients (pts) with "stable" DMR were comparatively analyzed both by qPCR, according to the last International Guidelines, and by dPCR (QS3D Digital PCR System). dPCR results were expressed as number of BCR-ABL1 copies/µl of reaction. This cohort of 118 pts were monitored for at least 3 time points every 3/4 months.

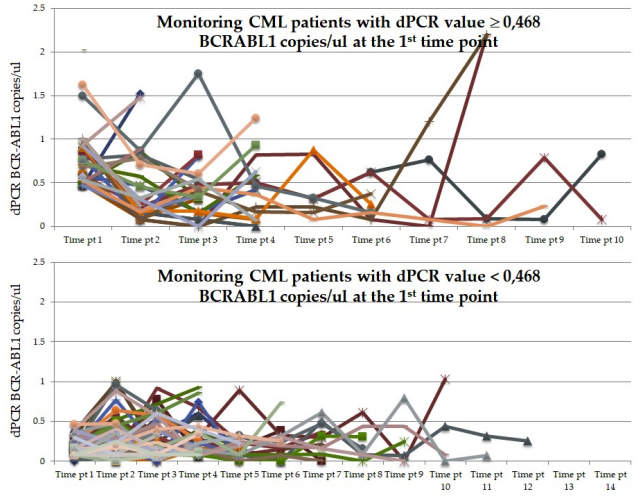

Figure 1. dPCR stability and TFR.

Among 118 pts, 87 discontinued TKI treatment: 35 (40%) had a stable MR4.0 while 52 (60%) had a stable MR4.5-5.0. These pts were analyzed for evaluating the positive predictive value (PPV) of qPCR vs dPCR for TFR using the previously described 0,468 BCR-ABL1 copies/µl cut-off (Bernardi S. *et al.*, J Mol Biom Diagn, 2017).

Results: At the enrolment, 50/118 (42%) pts resulted in MR4.0, 68/118 (58%) in MR 4.5-MR5.0. No significant difference were observed between these 2 groups both from clinical and follow-up patterns point of views. Among 87 pts who discontinued TKIs treatment, 24 lost the DMR (28%) after a median time of 6 months (qPCR PPV=72). They resulted 9/35 (26%) and 15/52 (29%) in stable MR4.0 and in stable MR4.5-5-0, respectively. The dPCR analysis on the 118 pts, according to the cut-off above mentioned, divided the pts in 2 groups: pts with dPCR value at the enrolment < and ≥468 BCR-ABL1 copies/µl. No significant clinical difference were observed between these 2 groups, except for age (p=0,009). The dPCR value distributions and the follow up of the pts revealed the capability of dPCR to detect heterogeneous BCR-ABL1 transcript levels patterns compared to the homogeneous levels detected by qPCR. The medians of the dPCR values were 0,258 (SD 0,215) and 0,395 (SD 0,419) for dPCR below and above the cut-off, respectively (p=0.022). Pts with a dPCR below the cut-off at the enrolment maintained a more stable and lower level of MRD than those with dPCR above the cut-off (Figure 1). Among the group of pts who discontinued TKI treatment, 55/87 (63%) and 32/87 (37%) had a dPCR < and ≥ 468 BCR-ABL1 copies/↘l at the time of discontinuation, respectively. 10/55 (18%) with dPCR below the cut-off and 14/32 (38%) with dPCR above the cut-off at the time of discontinuation, lost the DMR (dPCR PPV=82).

Conclusions: The results suggest that dPCR better identifies pts with stable DMR and eligible for TKI discontinuation. Furthemore, the dPCR PPV was higher than qPCR PPV in this cohort presenting a qPCR PPV (72%) higher than what reported in literature (PPV 40-60%).

PO055

DIGITAL DROPLET PCR AS A METHOD TO BETTER IDENTIFY CHRONIC MYELOID LEUKEMIA PATIENTS CANDIDATES TO TREATMENT-FREE REMISSION

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Introduction: Tyrosine kinase inhibitors (TKIs) discontinuation, in patients with chronic myeloid leukemia achieving a deep molecular response (DMR) would be an innovative strategy in order to reduce long-term toxicity. Several studies have reported that about 40% of patients maintain the response and reach a treatment free-remission. BCRABL1 transcript quantification is usually performed by RQ-PCR: digital droplet PCR (ddPCR) demonstrated a lower limit of detection (LOD) and quantification (LOQ) than traditional method and it could be useful to better identify CML patients candidates for discontinuation. The aim of this study was to evaluate ddPCR versus RQ-PCR in order to verify the advantage in terms of LOD and LOQ, to validate the test and to propose the quantification of residual transcript amounts as an alternative test prior to discontinuation.

Methods: We tested RNA samples from 50 patients in undetectable and stable MR4.5 according to IS evaluated by RQPCR. The ddPCR assay was carried out in triplicate using the Biorad-QX100 platform. We used the same primers and probes used in the standard RQ-PCR reaction. Each reaction mixture was partitioned into 20,000 droplets and then amplified. Cycled droplets were read in the QX200 droplet-reader and analysis was performed using the QuantaSoft software. Results were expressed as the absolute number of BCR-ABL1 copies. Samples yielding a minimum of 1 positive droplet scored as positive. The samples were tested a second time to confirm the

Results: There were 25 males and 25 females, median age was 63.4 years [37.5-85.5], with a median time of treatment of 8.9 years [1.4-14.4]. Forty-one patients had been treated with imatinib, while 9 patients had received second generation TKIs. In 34 (68%) patients, ddPCR confirmed the negativity of the samples, whereas in 16 (32%) a positivity was detected in at least 1 droplet with a median of 0.52 [0.22-2] BCR-ABL1 copies. We did not identify significant differences in baseline clinical features between ddPCR-positive and negative patients. No differences were revealed according to Sokal risk or in the type of TKI used as first-line treatment to reach a DMR between the two groups. Ten and 8 patients had been previously exposed to IFN-α in the ddPCR-positive

and ddPCR-negative groups, respectively. The duration of stable DMR and the median duration of treatment were similar in the ddPCR-positive and negative groups.

Conclusions: Our results suggest that ddPCR, compared to RQ-PCR, could be superior in terms of quantification of low levels of residual disease without the need of a calibration curve, in the same clinical conditions. In our cohort, considering any ddPCR positivity we could detect 34% of patients with a likely presence of minimal residual disease, suggesting that this tool increases the likelihood of identifying patients who could (or not) attempt TKI discontinuation within patients with an undetectable MR4.5, independently of the median time of treatment and of a stable DMR.

PO056

EVIDENCE OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA CLONES IN SOME PATIENTS WITH MYELOPROLIFERATIVE NEOPLASMS AND SIGNS OF HAEMOLYSIS OR UNEXPLAINED ANEMIA

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Background: The association between myeloproliferative neoplasms (MPN) and paroxysmal nocturnal hemoglobinuria (PNH) has been reported anecdotally. We aimed at evaluating prevalence and characteristics of PNH clones in a monocentric series of MPN patients with hemolysis and/or variations of their disease phenotype.

Methods: Patients enrolled in the study had diagnosis of MPN (according to WHO 2016 criteria) and either signs of haemolysis (LDH≥1.5 times above upper normal levels, reduced haptoglobin, reticulocytosis) or development of anemia in the context of an otherwise stable disease. Patients with progressive splenomegaly were excluded from the study. After informed consent, peripheral blood samples were used for multiparametric flow cytometric analysis on the basis of the expression of fluorescent inactive aerolysin (FLAER) and the GPI-anchored protein CD59 on RBCs, CD14 on monocytes, and CD24 on granulocytes, respectively. Samples were considered positive if PNH cells were ≥0.1% of total blood subpopulations. Clinical data were collected at diagnosis and at the time of the PNH assay.

Results: Thirty-six patients were studied (median age at diagnosis: 63 years, range 26-80), with Essential Thrombocythemia (ET, n=8), Primary Mielofibrosis (PMF, n=22), post-ET Myelofibrosis (post-ET MF, n=4), and MPN, unclassifiable (n=2). Flow cytometry identified a PNH clone in 3 patients, including 0.1-93% of RBCs, 0.1-89% of monocytes and 0.2-92.6% of granulocytes. Patients with evidence of PNH clones were affected by either JAK2V617F-mutated PMF, type I CALR-mutated PMF or JAK2V617F-mutated post-ET MF, respectively. Two out of 3 patients were receiving ruxolitinib treatment at the time of study inclusion. Mean hemoglobin level was not different in patients carrying or not PNH clones (10.37±1.85 vs 10.46±1.83, respectively), and reticulocyte count and LDH levels did not discriminate between patients with or without PNH cells. However, the size of PNH clone correlated to the degree of anemia (e.g. the patient with 93% PNH RBCs was the only who developed a transfusion-dependent anemia and had a sudden 3-fold increase in LDH level).

Conclusions: These preliminary data show that some MPN patients with signs of haemolysis or unexplained anemia may display PNH clones. It is not clear whether this evidence depends on the anti-JAK1/JAK2 treatment or is an intrinsic feature of the disease; consequently, a multicenter study is ongoing to correlate the presence of PNH clones in MPN patients with clinical characteristics, including thrombotic manifestations and type of treatment. In addition, genomic studies will be carried out to elucidate the clonal background of these patients.

PO057

ASSOCIATION OF PLK1 AND AURORA KINASE INHIBITORS WITH WEE1 INHIBITORS: A NOVEL THERAPEUTIC APPROACH FOR ADVANCED PHASES-CML

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Introduction: Polo-like kinases (PLKs) and Aurora kinases (Aks) are often overexpressed and deregulated in cancer cell, thus contributing to uncontrolled cell proliferation and consequent genomic instability. Chronic myeloid leukemia (CML) is a myeloproliferative disorder of hematopoietic stem cell. Despite the striking success of tyrosine kinase inhibitors (TKIs) therapy, a small proportion of CML patients may still progress from the chronic phase (CP) to the accelerated phase (AP) and eventually to the blast crisis (BC). This is likely due to cooperating molecular events additional to the initial t(9;22) translocation. In this study, a new therapeutic strategy based on AKA or Plk1 inhibition with PHA-739358 (Danusertib) or BI6727 (Volasertib), associated with Wee1 inhibition with AZD1775 was evaluated in K562 cells sensitive (K562-S) and resistant (K562-R) to imatinib and in primary cells from CML patients in BC.

Methods: Protein expression and activation was assessed by Western Blotting. Apoptotic cell death was quantified by annexin V/propidium iodide staining and flow cytometry. Cell cycle progression were evaluated by flow cytometry. Drug cytotoxicity in ex vivo experiments was evaluated by clonogenic assays in 1 healthy donor and in 3 CML patients in BC.

Results: Both Danusertib and Volasertib (0.5 μM for 24h) showed cytostatic and cytotoxic effects in CML cells by inducing G2/M- phase arrest and apoptosis. They caused a dose- and time-dependent reduction of the G0/G1 cell fraction and an increase of the G2/M fraction. Cell cycle arrest was associated with increased levels of phospho p-Chk1 and p-Chk2, p-cyclin B1, p-cdc2 and p-Wee1. Using a Wee1 inhibitor after 24h treatment with Danusertib and Volasertib 0.5 μM, when cells were arrested in G2 phase and Wee1 was overexpressed and hyper-activated, resulted in a synergistic inhibition of cell viability in both K562-S and -R. AZD1775 combined with either Danusertib or Volasertib caused a time-dependent increase of annexin-V-positive cells by activating the mitochondrial apoptotic pathway as reflected by an increment of Bax expression and induction of the cleavage of caspase-3, -9 and PARP. Moreover, both drug combinations induced a significant increase of the DNA double-strand break marker γH2AX, suggesting that Wee1 inhibition promotes mitosis and genomic instability by forcing the cells through successive replication cycles, ultimately resulting in apoptosis from mitotic catastrophe. Finally, clonogenic assays performed by using CD34+ progenitors from 3 BC CML patients, showed that PLK1 or AKs inhibition, associated with Wee1 inhibition, reduce the clonogenic activity of the CD34+ compartment in a synergistic way.

Conclusions: Our data indicate that PLK1 and AKs inhibitors associated with WEE1 inhibition display the potential for being further explored in innovative clinical trials aimed to improve the outcomes of patients with CML in blast crisis, or resistant to multiple lines of TKI therapy.

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PO058

POLYMORPHISMS IN MULTIDRUG RESISTANCE TRANSPORTER GENES MAY AFFECT THE LOSS OF MOLECULAR RESPONSE TO NILOTINIB IN CHRONIC MYELOID LEUKEMIA PATIENTS

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Background: Despite the high efficacy of Nilotinib in chronic myeloid leukemia (CML), there is still a significant proportion of patients who fail to maintain a major molecular response (MMR, MR3). For those patients, treatment-free remission once in deep molecular response (DMR), which is the final goal of nilotinib treatment, will never be an achievable endpoint. Little is known about the impact of pharmacogenetic variability in influencing MMR/DMR. It has been reported that ABC multidrug transporters, responsible for multi-drug resistance, interact with tyrosine kinase inhibitors (TKIs). However, data are missing about how genetic variants in ABC genes may modify pharmacological proprieties of second generations TKIs.

Aims: We investigated the impact of 5 single nucleotide polymorphisms (SNP) in three ABC transporter genes, namely ABCC1, ABCC2, and ABCB1, on achieving and maintaining molecular response (MMR, DMR) in CML patients treated with nilotinib.

Methods: We prospectively genotyped 95 CML patients treated at 7 Italian sites. The following SNPs in ABC multi-drug transporter genes were studied: ABCC1rs212090, ABCC2rs3740066, ABCC2rs4148386, ABCC2rs1885301, and ABCB1rs13435. Genomic DNA was analyzed by High Resolution Melting assay and pyrosequencing. All patients gave informed consent before entering the study, that was approved by Institutional Ethical Committees. Hardy-Weinberg equilibrium was verified for all examined SNPs. Difference in genotype and allele's distributions among the patients and the associations of the various genotype with response, resistance o lost of response to Nilotinib were determined using the Fisher's exact test. The associations between SNPs status and progression free survival (PFS) were assessed using Kaplan-Meier method and Log-rank test.

Results: MR3, MR4 and MR 4.5 were achieved by 89%, 57% and 49% of patients, respectively. Analysis of the difference in genotype distribution and alleles frequencies in responders and non-responders to Nilotinib showed that ABCC2 3972C>T SNPs (rs3740066) significantly impact on the loss of MR3, in dominant and codominantmodel (P=0.02 and P=0.01 respectively). Multivariate analysis in ongoing.

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PO059

DISCONTINUING TREATMENT IN CHRONIC MYELOID LEUKEMIA PATIENTS WITH MAJOR MOLECULAR RESPONSE: A REAL-LIFE EXPERIENCE

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Objectives: Discontinuation of TKI therapy is now feasible for some patients with chronic myeloid leukemia (CML) with deep and longstanding molecular response (MR4/4.5), and around 40 to 60% of these patients remain off-treatment. Few patients who stopped treatment after reaching major molecular response (MMR/MR3), but not MR4, have been reported (Michor F *et al.*, Nature 2005; Benjamini O *et al.*, Leuk Lymphoma 2014): all of them subsequently lost this molecular response. So, patients with MMR are not generally candidates for treatment discontinuation, and therapy withdrawal in this setting is usually reserved to severe toxicities or pregnancy. As many CML patients are not able to obtain a stable MR4/4.5, we intend to examine the outcome of patients who stopped TKIs when in MMR.

Methods: In the setting of a retrospective analysis we collected data about italian CML patients aged ≥ 18 years, who had been treated with TKIs and discontinued the treatment being in deep molecular response. A sub-analysis was performed on a group of patients who stopped treatment in stable MR3.

Results: We report 10 patients who stopped TKI treatment while in MR3; the main cause of discontinuation was pregnancy (4 female patients) or pregnancy planning (2 males); 3 patients stopped for toxicity and one case for patient's request. Six patients were in first line treatment (Imatinib in 5 and Nilotinib in one), four were in second line (Dasatinib in 3 and Nilotinib in one case). Three patients remained in MR3 without restarting any TKI; they were all low Sokal risk and they were exposed only to first line Imatinib. All of these three were alive and in MR4/4.5 at the last follow-up, 21.6, 76.3 and 99.8 months (mos) after stop, respectively (mean 65.9 mos). Mean time of any TKI treatment before stopping TKIs was 69.1 mos (range 17.1-146.7) in all patients, 93.3 mos (range 38.3-146.7) in the three patients still off-treatment, and 58.7 months (range 17.1- 90.7) in the patients who lost MMR and restarted treatment In 7 patients who restarted treatment, mean overall time off-therapy was 14.9 mos (range 3-33.3) and mean follow-up after stop was 30.5 mos (range 18-62).No death or progression occurred; two patients lost CCyR; all regained MMR or better after restarting treatment.

Conclusions: In a series of ten patients who stopped TKIs treatment with a stable MMR, three patients were able to maintain the molecular response without restarting treatment. A long exposure to Imatinib and low Sokal risk were common features of these patients. In all cases, including patients who restarted TKIs, discontinuation was not associated with progression and response was again obtained on treatment. Collecting data about safety of discontinuation or prolonged suspension of TKIs in MR3 cases is relevant as many CML patients planning pregnancy or dealing with toxicity may require to stop TKI treatment before obtaining a deep molecular response.

PO060

BOSUTINIB *VS* IMATINIB FOR NEWLY DIAGNOSED CHRONIC MYELOID LEUKEMIA (BFORE TRIAL): 24-MONTH EFFICACY UPDATE AND RESULTS BY 3-MONTH BCR-ABL1 TRANSCRIPT LEVELS

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Introduction: Bosutinib is approved for newly diagnosed chronic phase (CP) chronic myeloid leukemia (CML) and CML resistant/intolerant to prior therapy.

Methods: In the ongoing phase 3 BFORE trial (NCT02130557), 536 patients (pts) with CP CML were randomized 1:1 to 1st-line bosutinib (n=268) or imatinib (n=268 [3 untreated]). Efficacy was analyzed after ≥24 mo of follow-up in the intent-to-treat (ITT) population and by 3-mo BCR-ABL1 transcript levels (≤10% vs >10%) in evaluable pts (≥3000 ABL transcripts [bosutinib: n=248; imatinib: n=253]). Baseline and on-treatment time-dependent covariates were analyzed with a proportional subdistribution hazards model predicting time to initial major molecular response (MMR). No adjustment for multiple comparisons was made.

Results Higher molecular response (MR) rates were seen for bosutinib vs imatinib at 12 mo; the between-arm difference in MMR rate was retained at 24 mo, but differences in rates of deeper MRs (MR4/MR4.5) were smaller (Table). Times to MR were shorter for bosutinib vs imatinib. In an event-free survival (EFS) analysis, 14 pts in the bosutinib arm and 17 in the imatinib arm had on-treatment events, including transformation to accelerated/blast phase in 6 and 7 pts, respectively. 71% vs 66% remained on bosutinib vs imatinib. A higher proportion of evaluable pts in the bosutinib vs imatinib arm had BCR-ABL1 transcripts ≤10% at 3 mo (80.6% vs 60.5%). MMR rate at 24 mo was higher in pts with 3-mo BCR-ABL1 transcript levels ≤10% vs >10% in the bosutinib (73.5% vs 33.3%; P<.0001) and imatinib (68.6% vs 30.0%; P<.0001) arms. In a multivariable model in the ITT population, 3-mo BCR-ABL1 transcripts ≤10% (vs >10%/not evaluable) was a predictor of time to MMR (bosutinib: hazard ratio [HR]=3.353, P<.0001; imatinib: HR=2.683, P<.0001). Due to relatively short follow-up, there were not enough events to detect differences in EFS or overall survival by 3-mo BCR-ABL1 transcript level with bosutinib.

Conclusions At 24 mo, a higher MMR rate was maintained with bosutinib vs imatinib, supporting 1st-line bosutinib use for CP CML. BCR-ABL1 transcript level at 3 mo appears to be a predictor of the time to obtain an MMR, both with bosutinib and with imatinib.

Acknowledgments: The study is sponsored by Pfizer. Medical writing support was provided by Joanna Bloom, PhD, of Engage Scientific Solutions, and funded by Pfizer.

Table 1.

	ITT Population		
	Bosutinib n=268	Imatinib n=268	*P*
Cumulative (any time on-treatment), %			
MMR	68.7	59.3	.024
MR[4]	39.9	31.3	.040
MR[4.5]	25.7	19.0	.063
MMR, %			
at 12 mo	46.6	36.2	.013
at 24 mo	61.2	50.7	.015
MR[4], %			
at 12 mo	20.5	11.6	.005
at 24 mo	32.8	25.7	.073
MR[4.5], %			
at 12 mo	7.5	3.0	.020
at 24 mo	13.1	10.8	.428
Time to response (based on cumulative incidence), HR*			
MMR	1.37		.004
MR[4]	1.39		.025
MR[4.5]	1.42		.054
Transformation to accelerated/blast phase (cumulative incidence), %			
at 12 mo	1.9	2.6	
at 24 mo	2.2	2.6	
Kaplan-Meier overall survival rate, %			
at 12 mo	99.6	98.1	
at 24 mo	99.2	97.0	

*Bosutinib vs imatinib; HR >1 indicates shorter time to response for bosutinib

PO061

LONG-TERM EFFICACY AND CARDIAC, VASCULAR AND HYPERTENSION SAFETY OF BOSUTINIB IN PREVIOUSLY TREATED PATIENTS WITH PHILADELPHIA CHROMOSOME–POSITIVE LEUKEMIA

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Introduction: Bosutinib is approved for patients (pts) with chronic, accelerated or blast phase (CP, AP or BP) Philadelphia chromosome–positive (Ph+) chronic myeloid leukemia (CML) resistant/intolerant to prior therapy and newly diagnosed pts with CP CML.

Methods: A phase 1/2 study (NCT00261846) assessed bosutinib in Ph+ pts with CP CML resistant/intolerant to imatinib (CP2L) or imatinib plus dasatinib and/or nilotinib (CP3L) and those with AP/BP CML or acute lymphoblastic leukemia after imatinib (advanced [ADV]). We report long-term bosutinib efficacy and cardiac, vascular and hypertension safety (treatment-emergent adverse event [TEAE] clusters from prespecified MedDRA terms and queries) in this study and its ongoing extension study (NCT01903733).

Table 1.

Pts in TEAE clusters, n (%)	CP2L n=284	CP3L n=119	ADV n=167	Total N=570
Cardiac	40 (14)	18 (15)	22 (13)	80 (14)
Grade ≥3	19 (7)	9 (8)	7 (4)	35 (6)
SAE	18 (6)	7 (6)	10 (6)	35 (6)
Leading to bosutinib withdrawal	1 (<1)	5 (4)	4 (2)	10 (2)
Drug-related	14 (5)	13 (11)	7 (4)	34 (6)
Most frequent TEAEs				
Pericardial effusion	9 (3)	7 (6)	6 (4)	22 (4)
Atrial fibrillation	8 (3)	7 (6)	2 (1)	17 (3)
Cardiac failure congestive	8 (3)	4 (3)	3 (2)	15 (3)
Vascular	27 (10)	9 (8)	15 (9)	51 (9)
Grade ≥3	16 (6)	7 (6)	11 (7)	34 (6)
SAE	19 (7)	7 (6)	12 (7)	38 (7)
Leading to bosutinib withdrawal	3 (1)	2 (2)	2 (1)	7 (1)
Drug-related	5 (2)	1 (1)	1 (1)	7 (1)
Most frequent TEAEs				
Angina pectoris	9 (3)	1 (1)	0	10 (2)
Coronary artery disease	4 (1)	2 (2)	2 (1)	8 (1)
Acute myocardial infarction	2 (1)	1 (1)	2 (1)	5 (1)
Hypertension	29 (10)	11 (9)	13 (8)	53 (9)
Grade ≥3	10 (4)	3 (3)	5 (3)	18 (3)
SAE	3 (1)	0	1 (1)	4 (1)
Leading to bosutinib withdrawal	0	0	0	0
Drug-related	5 (2)	3 (3)	0	8 (1)
Most frequent TEAEs				
Hypertension	26 (9)	10 (8)	12 (7)	48 (8)
Blood pressure increased	2 (1)	0	1 (1)	3 (1)

Results: After ≥7 y of follow-up overall (CP2L: 8y; CP3L/ADV: 7 y), 24%, 8% and 5% of CP2L (n=284), CP3L (n=119) and ADV (n=167) pts, respectively, were still receiving bosutinib. Treatment discontinuations at >5 y on treatment due to disease progression (n=8, 1 and 1), TEAEs (n=7, 5 and 2), and death (n=6, 1 and 0) were relatively low for CP2L, CP3L and ADV pts, respectively. Of CP2L (n=262), CP3L (n=112), AP (n=72) and BP (n=54) pts with a valid baseline assessment, respectively, major cytogenetic response (MCyR) was achieved by 60%, 41%, 40% and 37% and complete cytogenetic response (CCyR) by 50%, 32%, 31% and 28%; Kaplan-Meier (KM) probability of maintaining MCyR was 65%, 64%, 41% and 0, and maintaining CCyR was 61%, 45%, 40% and 0 for responders at the minimum follow-up. Cumulative incidence of on-treatment progression or death was 23% in CP2L, 25%

in CP3L, 39% in AP and 63% in BP pts; KM overall survival was 79%, 72%, 59% and 23% at the minimum follow-up. Rates of TEAEs, serious adverse events (SAEs), drug withdrawals and drug-related TEAEs in cardiac, vascular and hypertension clusters were generally similar across cohorts (Table 1); 4 (3 CP2L/1 ADV), 10 (1 CP2L/2 CP3L/7 ADV) and 0 TEAEs led to death, respectively.

Conclusions: Bosutinib showed long-term efficacy in previously treated pts with CML. Incidence of cardiac, vascular and hypertension TEAEs was low and events infrequently led to treatment withdrawal.

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Myelodysplastic Syndromes

PO062

IRON SUPPORT REDUCES ERYPTOSIS LEVEL AND ENHANCES ERYTHROPOIETIN EFFECTIVENESS IN REFRACTORY ANEMIA WITH HIGH RETICULOCYTE COUNT AND TRANSFERRIN SATURATION BELOW 30%

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Introduction: Eryptosis (erythrocyte apoptosis) is an enhanced phenomenon in myelodysplastic syndromes(MDS) positively related to reticulocyte count and triggered by iron deficiency. Iron support is not recommended in MDS,also at low risk, in which iron chelation is a pivot of treatment.Aim of this study is to see if iron support enhances erythropoietin effectiveness in refractory anemia with high reticulocyte count, transferrin saturation below 30%, regardless of ferritin level

Patients and methods: Between july 2015 and december 2018, 40 patients affected by refractory anemia with IPSS low-risk were studied.

Median follow-up was 16 months (R12-28). Patients were randomized 1:1 to receive in group A (with corrected reticulocyte count <2.5%, transferrin saturation >30%) sucrosomial iron 30 mg 2 tablets orally/day +erythropoietin 40000 IU sc/t.i.w + calcium levofolinate 7.5 mg/day orally + Vitamin B12: 400 mg/day orally. In group B (with corrected reticulocyte count >2.5%, transferrin saturation <30%)patient received sucrosomial iron 30 mg 2 tablets orally/day + alpha erythropoietin 40000 IU sc/t.i.w. + calcium levofolinate 7.5 mg/day orally + Vitamin B12: 400 mg/day orally. In group A median age was 75 years (R70-81), M/F: 8/12.

In group B median age was 72 years (R68-77), M/F: 10/10. Caryotype was normal in group A and B patients. Median level of haemoglobin was 9.5 g/dl in group A (R9-10) and 8.9 g/dl (R8.5-10) in group B. Median ferritin level was 900 ng/ml in group A (R890-1100) and 800 ng/ml g/dl (R750-950) in group B. Eryptosis was measured with cytosolic Ca2+, utilizing Fluo3 fluorescence, after cells staining with the Fluo-3 AM dye (Biotium, Hayward, USA). Levels of eryptosis at start of treatment were higher in group B (50% VS 35%).

Results: Group A patients increased Hb level of 1 g/dl after a median time of 6weeks (R4-7), 11 g/dl after 3 month. Group B patients increased Hb level of 1 g/dl after a median time of 4weeks (R3-5), 12g/dl after 3 month. Median ferritin level was 1100 ng/ml in group A (R950-1300) and 850 ng/ml (R800-900) in group B. After treatment levels of eryptosis were higher in group A (38%vs10%) .

Conclusions: Iron support enhances erythropoietin effectiveness in refractory anemia with high reticulocyte count, transferrin saturation below 30% probably reducing level of eryptosis involved in ineffective erythropoiesis.

PO063

A RARE CASE OF MYELODYSPLASTIC SYNDROME WITH CHROMOSOMAL REARRANGEMENT INVOLVING 11Q23 LOCUS, BUT NOT MLL GENE

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Introduction: Chromosome 11q23 traslocations, resulting in MLL (KMT2A) rearrangement, have been well characterised in acute myeloid leukemia (AML), acute lymphoblastic leukaemia (ALL); less commonly with myelodysplastic syndromes (MDSs); rarely with T-Lymphoblastic leukemia (T-ALL) and chronic myelomonocytic leukemia (CMML). However, rare cases have been reported in which 11q23 translocation

was not associated with MLL rearrangement (11q23+/MLL-). We report a case of a patient, 69-years-old male, with diagnosis of MDS in which the 11q23 translocation was detected but not the MLL rearrangement.

Methods: Laboratory examination revealed a white blood cell count of $5.43x10^3$/µl, $2.02x10^3$/µl red blood cells and $320x10^3$/µl platelets. The haemoglobin concentration was 8.5 g/dl, haematocrit 25%, and mean corpuscular volume 123 fl. Peripheral blood smears, bone marrow (BM) aspirate smears were reviewed. Blast percentage, BM cellularity and background dysplasia were assessed. Convetional Chromosomal analysis was performed on G-banded metaphase cells prepared from unstimulated BM aspirate culture using standard techniques. Fluorescence *in situ* hybridization (FISH) was performed with MLL dual-colour break-apart probe, and LSI CSF1R/D5S23, D5S721 dual colour brobe, (Abbott Molecular/Vysis), on freshly harvested BM cells (metaphase or interphase).

Results: The morphological examination of the peripheral blood showed hyposegmented neutrophils, some activated lymphocytes, anisopoikilocytosis of the red blood cells, anisocytosis of the platelets. The analysis of bone marrow aspirate showed percentage of blasts of less than 1%. The karyotype reveled two cell lines: a first clone was 46, XY, t(2;11)(p21;q23-24)[6] while a second clone was 46,XY, t(2;11)(p21;q23-24)/del(5q)(q15q33)[4]. Analysis by FISH was found to be negative for MLL rearrangement, while the 5q deletion was confirmed.

Conclusions: A few cases with 11q23+/MLL- have been reported in patients with CMML, MDS, B-ALL and AML. Frequently the t(2;11)(p21;q23) was been associated with del(5q) as in our patient. The patients with 11q23+/MLL- appears to be associated with clonal evolution/disease progression in acute myeloid leukaemia. However, due to the small number of cases and the heterogeneity of patients reported in the literature, the prognostic significance is to be defined.

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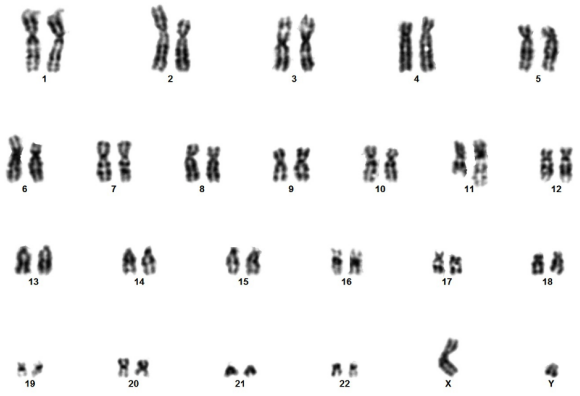

Figure 1. Karyotype 46, XY, t(2;11)(p21;q23-24).

PO064

CLINICAL UTILITY OF FLOW CYTOMETRY SCORE AND CD34+ CELL COMPARTMENT EVALUATION IN PATIENTS WITH MYELODYSPLASTYC SYNDROME (MDS)

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Introduction: Currently, flow cytometry (FC) is a valuable additional tool for the diagnosis of MDS. Most of the designed FC scores are based on the evaluation of the myelomonocytic abnormalities with a median sensitivity of 75% for identifying MDS. Several studies have also reported in approximately 40% of patients with MDS, expression of lineage infidelity markers (LIM) on CD34+ cell compartment.

Methods: We evaluated in 63 bone marrow (BM) samples (46 patients with MDS and 17 controls): 1) expression of LIM CD7, CD56, CD2, CD5, CD25 on CD34+ cells; 2) FC Ogata score based on CD34+ myeloblasts (%), CD34+ B-progenitors (%), myeloblast CD45 expression and granulocyte to lymphocyte SSC peak channel ratio. Diagnosis of MDS was highly suggestive for FC score ≥ 2. Immunophenotypical analysis was performed using 8-color flow cytometry on total nucleated bone marrow cells after lysis of erythrocytes. A minimum of 10^3 CD34+ cells was collected. Results were compared with control BM samples. Aberrant expression of LIM was defined as more than maximum expression of that specific antigen in control BM.

Results: Median age was 74 years (range 44-88) and 24 patients (52%) were female. According to 2016 WHO criteria: 17% were MDS with single lineage dysplasia, 6.5% MDS with ring sideroblasts, 4.5% MDS with isolated del(5q), 35% MDS with multilineage dysplasia, 28% MDS with excess blasts, 9% chronic myelomonocitic leukemia. Karyotype was evaluable in 42 patients and, according to revised International Prognostic Scoring System (r-IPSS) cytogenetic scoring system, 88% had good risk, 9.5% intermediate and 2.5% poor risk karyotype. R-IPSS, was estimated in 40 patients and 10%, 42.5%, 20%, 17.5%, 10%, were very low, low, intermediate, high, very high risk, respectively. In control BM samples FC score was always < 2 (specificity 100%), whereas in MDS patients FC score was ≥ 2 in 33 patients (sensitivity 72%). Overall, LIM were found in 32 patients (70%). Aberrant expression of 1, 2 and 3 antigens was detected in 15 (47%), 14 (44%) and 3 (9%) patients, respectively. The incidence of CD7, CD56, CD2, CD5 and CD25 expression was 41%, 33%, 2%, 17% and 26%, respectively. Considering 13 patients with FC score <2, expression of LIM was detected in 8 (61%). Therefore, by combining FC score with CD34+ cell compartment evaluation the sensitivity of test raised to 89%. Low FC score (0-2) was significantly associated with very low/low r-IPSS risk groups, normal karyotype and no detection of LIM (p=0.05, 0.04 and 0.03, respectively). Aberrant expression of 2-3 antigens was significantly more frequent in intermediate/high/very high than very low/low r-IPSS risk groups (73% vs 27%, respectively, p=0.01).

Conclusions: 1) Combined assessment of FC score and expression of LIM on CD34+ cell compartment improves diagnostic power of flow cytometry in MDS. 2) Low FC score and no expression of LIM are associated with good prognosis features.

PO065

APOPTOTIC AND DIFFERENTIATING EFFECTS OF THE POLY(ADP-RIBOSE) POLYMERASE INHIBITOR OLAPARIB IN MYELODYSPLASTIC SYNDROMES

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Introduction: Olaparib (AstraZeneca), a potent poly-ADP-ribose polymerase 1 inhibitor, is orally bioavailable, has addressable toxicities and has been lately approved as maintenance therapy in patients with recurrent BRCA-mutated ovarian and breast cancer. Myelodysplastic syndromes (MDS) affect mainly elderly people. Peripheral cytopenia, hematopoietic cell lineages dysplasia and tendency to evolve to acute myeloid leukemia are the main hallmarks of these diseases. To date, only few treatment options are available for patients with MDS and survival is still unsatisfactory.

Methods: Freshly isolated primary cells were obtained from 27 BM aspirates of adult patients with newly diagnosed MDS. Mononuclear cells were treated *in vitro* with graded concentrations of olaparib or decitabine for up to 7 days. Cell survival was studied by counting viable cells and used for calculation of drug inhibitory concentration 50 (IC50) values. Apoptosis was assayed using an annexin V/PI kit. Differentiation of MDS was analyzed by flow cytometry. Quantitative real-time PCR was carried out by SYBR Green technology. Total proteins were analyzed by western blot. Analysis of cytotoxicity synergism was performed by MTS assay.

Results: - A single treatment with olaparib exerted anti-proliferative effects in the majority of primary MDS MNC with IC50 values below or in the range of the plasma peak concentration reached in cancer patients. - Olaparib had a selective cytotoxic effect on myeloid hematopoietic cells, while sparing lymphocytes that are not part of the MDS disease. - BM cells from MDS patients are characterized by a decrease of neutrophil granularity and reduction of differentiation antigens or their ratios. Using flow cytometry, in cells treated with olaparib, we observed a significant reduction of the proportion of CD117+/CD123+ immature progenitors (11 out of 12 samples). Moreover, most MDS samples showed a significant induction of CD11b+/CD16+, CD13+/CD16+ and CD10+/CD15+ double positive cells, indicating differentiation towards mature granulocytes. - The differentiation of hematopoietic cells induced by olaparib was confirmed by a dose-dependent increase of RNA and protein expression of the transcription factors PU.1 and CEBPA, which are involved in granulocytic and monocytic differentiation. - We then tested the effects of decitabine, which is currently used in MDS therapy. Equitoxic decitabine and olaparib concentrations, exhibited synergistic cytotoxic effects in primary MDS culture. Moreover, the percentage of CD117+/CD123+ progenitors decreased, while CD11b+/CD16+ mature neutrophils increased with the drug combination as compared to treatment with single agents.

Conclusions: In summary, our preclinical data suggest that olaparib induces cytotoxic and differentiating effects in primary MDS cells. Decitabine and olaparib combination might have a therapeutic potential in MDS patients unfit for chemotherapy or bone marrow transplantation.

Acute Leukemia 2

PO066

TYROSINE KINASE INHIBITORS (TKI) IN RELAPSED/REFRACTORY (RR) PATIENTS WITH FLT3-ITD POSITIVE ACUTE MYELOID LEUKEMIA (AML) CONFER BETTER SURVIVAL THAN CHEMOTHERAPY, DUE TO A BETTER SAFETY PROFILE

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Background: Approximately 20-30% of AML patients harbor internal tandem duplication (ITD) of FLT3 gene. Several drugs have been developed to inhibit FLT3. However, R/R FLT3-ITD AML patients still represent an unmet clinical need.

Aims: Our aim is to assess outcome, safety and duration of hospitalizations in two retrospective groups of patients treated with TKIs or chemotherapy, respectively.

Methods: We retrospectively collected and analyzed clinical and biological data of 58 consecutive FLT3-ITD AML patients, referred to or treated at our Institution from 2004 to 2017. Patients received chemotherapy (3+7 like regimens; 3+7 like regimens with the addition of a third agent; fludarabine based regimens) and/or single agent TKIs (Sorafenib, Ponatinib, Quizartinib, Gilteritinib, Midostaurin).

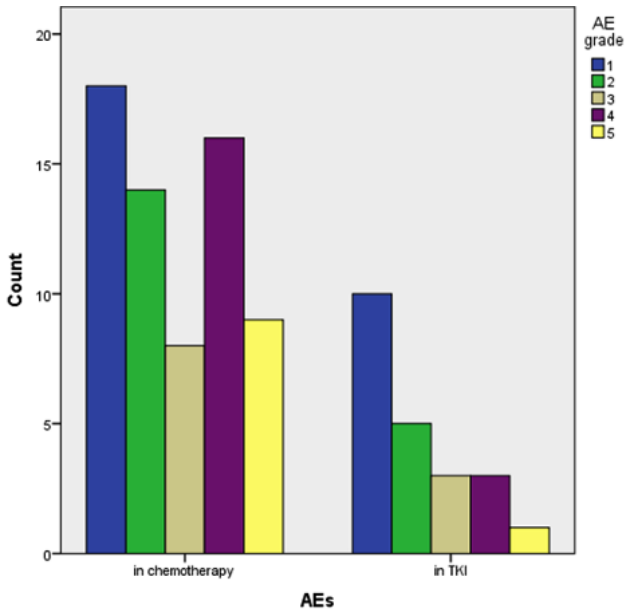

Figure: number and grade of adverse events in patients undergoing TKI compared with patients treated with chemotherapy (p=.049).

Figure 1.

Results: We compared patients who received at least once in their life, as salvage treatment, a TKI inhibitor ("TKI" group; N=36) with patients that were treated exclusively with conventional cytotoxic ("conventional group"; N=22). There were no significant differences in patient age, white blood cells count, platelet count and ELN risk at diagnosis between the two groups. Fifty-one out of 58 patients (86%) relapsed after (N=22; 36%) or were refractory (N=29; 50%) to the first course of induction

chemotherapy. Second-line therapy included salvage chemotherapy (N=32/51, 63%), a TKI (N=12/51, 23%) or best supportive therapy (N=14%, 7/51); 24 patients received a TKI in 3rd or further relapse.

Standard chemotherapy compared with TKIs did not show an increased efficacy in terms of CR (25% vs 16.7%), and it was not a better bridge-to-transplant option (56.2% vs 43.7%). However, among R/R patients, we observed an advantage in terms of OS for patients of the "TKI" group compared with "conventional" group (median OS from R/R of 10 months [95% CI, 5.89-14.12] and 4 months [95% CI, 3.12-4.90], respectively; p= .017). Patients in "TKI" group experienced a lower number of AEs during treatment with TKIs (1.63 mean AEs in each TKI line vs 3.03 mean AEs in each chemotherapy line, excluding stem cell transplant; p< .001). AEs during TKI therapy were less severe if compared with AEs during chemotherapy (grade III-IV 6/23 and grade V 2/23 with TKI; grade III-IV 24/66 and grade V 12/66 with chemotherapy; figure; p= .049). We also noted a trend toward less day spent in hospital per month by patients during TKI treatment, compared to patients treated with standard chemotherapy (including post chemotherapy remission period): 10.5 days and 16.7 days in the two groups, respectively.

Conclusiosn: Our study, even if in a retrospective set, reports a survival advantage of TKI in R/R FLT3 ITD AML patients. Such an advantage is due to the lower number and grade of AEs of "TKI" group. For their safety profile, TKIs are probably a better option to bridge patients to transplant, thanks to a lower risk of toxicity.

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PO067

ACUTE MYELOID LEUKEMIA CELLS PRODUCE INTERFERON GAMMA AND UPREGULATE IMMUNE TOLERANCE GENES IN LEUKEMIC MESENCHYMAL STROMAL CELLS

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Introduction: Mesenchymal stromal cells (MSCs) substantially contribute to the creation of hemopoietic niche by regulating hematopoietic stem cell (HSC) fate and have a unique immune-modulating capacity. In the leukemic milieu, the presence of MSCs constitutes a side effect, since MSCs not only favor leukemic cell survival, but they can also generate an immune-tolerant environment. Although recent findings have outlined a putative MSC role in hematological malignancy development, MSC-dependent mechanisms potentially supporting leukemia remain unclear. We hypothesize that neoplastic cells can induce in MSCs functional changes which turn the bone marrow (BM) in a leukemia-supporting milieu.

Methods: We isolated acute myeloid leukemia (AML) cells and AML-MSCs from the BM of AML patients. Next, we set up AML-MSC/AML cell co-culture experiments and we investigated gene expression in AML-MSCs and AML cells before and after co-cultures.

Results: Our microarray data indicated Interferon(IFN)-γ as un up-regulated gene in almost 40% of AML samples. Furthermore, preliminary analysis, indicated that IFN-γ-positive AML patients showed a better overall survival. Thus, we decided to deepen IFN-γ-dependent modifications in leukemic milieu. IFN-γ stimulating indoleamine 2,3-dioxygenase (IDO)1 enzyme expression is a well-known nodal modifier of MSC immune-modulatory properties. In AML-MSC/AML cell co-culture experiments, we confirmed microarray data and we found that AML cells produced IFN-γ. We also demonstrated that IDO1 is up-regulated in AML-MSCs after co-culture with AML cells. Moreover, an IFN-γ receptor neutralizing antibody reduced the AML cell-dependent up-regulation of IDO1 in MSCs. Finally, we found that AML-MSCs after co-culture with AML cells, i.e. after IDO1 up-regulation, were able to induce regulatory T cell. To gain insight in AML cell-dependent MSC modifications, we decide to analyze MSC expression of IFN-γ stimulated genes (ISG) such as PDL-1 and NOS2 which are known to regulate immunity and promote tolerance.In particular, we tested ISG expression in MSCs after co-cultures with IFN-γ positive or IFN-γ negative AML cells. We found that IFN-γ positive, but not IFN-γ negative AML cells, were able to induce PDL-1 and NOS2 in AML-MSCs. Thus, ISG expression profile in AML-MSCs after co-cultures with IFN-γ positive AML cells resulted similar to ISG expression profile of MSCs treated with recombinant IFN-γ.

Conclusions: Our data suggested that inflammatory signals produced by AML cells are able to modify MSC functions favoring an immune-tolerant and leukemia supporting milieu. Overall, our results would likely contribute to unravel MSC–dependent mechanisms promoting leukemia and will help to provide novel applications for drugs already under experimentation (e.g. IDO-inhibitors, Checkpoint inhibitors) to translate into more effective therapies in AML patients.

PO068

PRELIMINARY ANALYSIS OF THE PROGNOSTIC ROLE OF RAS PATHWAY MUTATIONS IN ADULT B-LINEAGE ACUTE LYMPHOBLASTIC LEUKEMIA (B-ALL) PATIENTS WITHOUT MAJOR FUSION GENES ENROLLED IN THE GIMEMA LAL1913 PROTOCOL

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Introduction: We recently showed that in B-ALL without major fusion genes (i.e. BCR-ABL1, ETV6-RUNX1, TCF3-PBX1 and KTM2A-r, defined B-NEG ALL) adolescents and adults carrying RAS mutations have an inferior survival (Messina et al, 2016). However, the prognostic impact of clonal and subclonal RAS pathway mutations in adult B-ALL patients enrolled in a pediatric-oriented, minimal residual disease (MRD)-driven trial has so far not been assessed. We aimed at investigating: i) the incidence of RAS pathway clonal and subclonal mutations; ii) the prognostic role of RAS mutations within JAK/STAT-WT cases, and iii) the correlation with clinico-biologic correlates (WBC, plts, age, BCR-ABL1-like profile) in B-NEG ALL patients enrolled in the GIMEMA LAL1913 clinical trial for adult BCR/ABL1-negative ALL patients.

Methods: The screening of RAS members (NRAS, KRAS, FLT3, and PTPN11) was performed in 88 B-NEG ALL patients (median age: 38.2 years, range 18.2-64.7) by next generation sequencing (NGS, Truseq custom amplicon kit; Illumina Miseq) and Variant Studio Software. Only variants satisfying the following criteria were retained: 1) exonic variants; 2) missense and truncating variants, 3) genotype quality equal to 100; 3) read-depth >100. All variants recognized as SNPs were excluded, unless a prognostic value was previously demonstrated.

Results: We detected a total of 58 RAS pathway mutations in 41 (46.6%) cases, with 8 cases displaying >1 mutated gene and 7 cases with >1 mutation targeting the same gene. The most frequently affected gene was NRAS, mutated in 18 (20.4%) cases, followed by KRAS, mutated in 14 (15.9%) with 87.5% of mutations targeting the hotspot G12-G13 and only 9.4% the hotspot Q61. FLT3 and PTPN11 proved mutated in 10 (11.4%) and 7 (7.9%) cases, respectively. A considerable proportion of mutations (30, 51.7%) were detected at the subclonal level (variant-allele frequency <15%). RAS pathway mutations were more frequent in non-BCR-ABL1-like (50.9%) than BCR-ABL1-like (33.3%) cases, though this difference was not statistically significant. Survival analysis

showed that patients carrying clonal RAS pathway mutations had an inferior overall survival (OS) than WT-cases (57.5% vs 83.5%, p=0.009). Consistently, disease-free survival (DFS) of RAS pathway mutated cases was lower than that of WT-cases (61.9% vs 83.4%, p=0.091). The survival of patients harboring subclonal mutations resembled that of WT cases; a broader cohort is required to confirm this finding.

Conclusions: This preliminary analysis of the GIMEMA LAL1913 clinical trial confirms that RAS pathway mutations identify a subgroup of B-NEG ALL patients that experience an inferior survival, thus the analysis of their mutational status should be included in the diagnostic workflow. Since mutated samples are sensitive to PI3K/mTOR, MEK or FLT3 inhibitors, these mutations may recognize patients eligible for alternative treatments.

PO069

ACUTE MYELOID LEUKEMIA BLASTS AND STEM PROGENITOR CELLS SHOW GENOMIC-DRIVEN AND FUNCTIONAL ALTERATIONS IN ENERGY, AMINO ACID AND LIPID METABOLISM

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Introduction: Altered cellular metabolism participates to AML pathogenesis, predicts response to therapy and can be targeted. The study aims to map potential connections between genetic lesions and metabolism in AML and identify metabolic alterations underlying druggable pathways.

Methods: WES was performed on 78 AML. Variants were called by MuTect and Varscan2. Metabolites were quantified by mass spectrometry of bone marrow (BM) cells (35 CD34+, 16 CD33+ AML), CD34+ cord blood and CD33+ healthy blood cells (n=21, Metabolon). Gene expression profiling was performed on 56 BM (AML and healthy donors, Affymetrix). Cell specific metabolic reconstructions derived with iMat and mCADRE algorithms were studied for data integration.

Results: Metabolism-related genes were mutated in 67% AML. The most represented pathways were lipid and nucleotide metabolism (mutations in the pyrimidine gene AK9 and the pentose phosphate pathway (PPP) gene H6PD); bioenergetics (respiratory chain genes NNT and ADCK3); amino acid metabolism (tryptophan-related gene IDO2); carbohydrate and glucose metabolism (altered PKM, HK3); response to stress (mutated SOD2). AML cells showed distinct metabolic profiles compared with healthy cells, with little differences between AML subgroups. The top 30 ranking biochemicals pointed to lipid, nucleotide and amino acid metabolism, in line with genomic data. The bioenergetic pathway was also altered in CD34+ AML, with reduced glycolytic metabolites (FDP, 3PG, PEP) and enzymes (GPI, PFKFB3) and Krebs cycle intermediates (citrate, aconitate, succinate, malate). The PPP metabolite S7P and intermediates of purine/pyrimidine synthesis were increased, suggesting elevated demands in DNA/RNA synthesis. Half AML cases displayed high 4-HNE-glutathione (15-50-fold), that correlated with GSH and GSSG levels and was indicative of elevated detoxification of 4-HNE. Moreover, CD34+ and CD33+ AML showed distinct glutamine/glutamate-dependence: increased NAAG and NAA in CD33+ AML, along with GLS upregulation: decreased NAAG and elevated glutamine in CD34+ AML. To investigate the relations between genomic and metabolic alterations, we searched for the most fitting metabolic model. Metabolites, reactions and genes annotated in BM, blood, CD14+ cell and monocyte subsystems by mCADRE were largely overlapping and shared a core with an iMat hematological model. 69% and 14% of altered metabolites and transcripts were mapped in the RECON model: of them, 31% and 69% metabolites and 28% and 26% genes, on average, were present in mCADRE and iMAT models, respectively.

Conclusions: Mutations of metabolic genes are common in AML. Genomic-driven and functional metabolic changes converge to common pathways. Dissecting the link between genomic and metabolic alterations may help define AML subtypes sensitive to antimetabolic therapies, as PPP inhibition and 4-HNE treatment.

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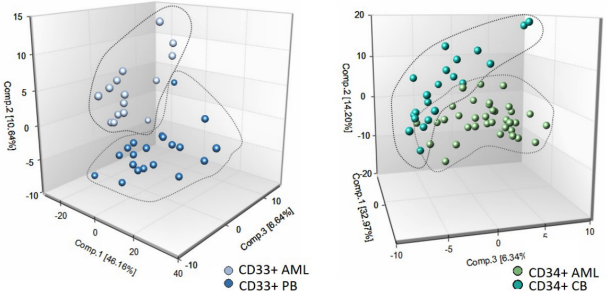
Principal Component Analysis discriminates AML cells from normal cells based on their metabolic profile.



Principal component analysis of metabolic data (300 metabolites) showing separation of AML cells (CD33+ blasts or CD34+ stem progenitor cells) from normal cells (CD33+ cells from peripheral blood of healthy donor and CD34+ cord blood cells, respectively).

Figure 1.

PO070

ROLE OF WNT/β-CATENIN-GSK3 SIGNALING PATHWAY IN NON-PROMYELOCYTIC ACUTE MYELOID LEUKEMIA CELLS RESPONSE TO CHEMOTHERAPY THROUGH MICROENVIRONMENT PROTECTION

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Introduction: Wnt/β-catenin-GSK3 signaling pathway as one of the most relevant signaling mechanisms in tumour biology. Aberrant Wnt/β-catenin signaling can be involved in neoplastic myeloid cell growth and the association of GSK-3 inhibitors with All-Trans Retinoic Acid (ATRA) significantly improves cell death in acute promyelocytic leukemia. However, little is known about the contribution of stromal Wnt/β-catenin-GSK3 signaling pathway to non-promyelocytic acute myeloid leukemia (AML) survival and chemoresistance.

Methods: Wnt/β-catenin-GSK3 expression were analyzed by western-blot and flow cytometry in primary non-promyelocytic AML blast cells (30 samples) and AML cell lines (HL60, THP1, U937), cultured alone or in presence of human-bone marrow mesenchymal stromal cells (hBM-MSCs) derived from healthy donors (HD-MSCs) or AML patients (PT-MSCs). AML cells cultured alone or in presence of hBM-MSCs were treated with Wnt/β-catenin or GSK3 inhibitors, including Niclosamide, PNU-74654, IWP-2 and LiCl, AR-A014418 and SB 216763, respectively, in association or not with Cytarabine (Ara-C) or Idarubicin. Mouse xenograft model of non-promyelocytic AML was obtained by caudal injection of the AML line U937 in NOD/Shi-scid/IL-2Rγnull mice (NOG). Cell viability was evaluated by Annexin-V/PI and MTT assay; proliferation was measured by CFSE dilution.

Results: The presence of total β-catenin was shown only in about 2/3 of primary AML samples analyzed, while the active form of β-catenin increased in AML samples in co-culture with hBM-MSCs, suggesting the possible involvement of Wnt/β-catenin signaling in the crosstalk

between stromal and AML cells. On the other hand, high expression of all GSK3 forms was found in AML samples, which were all down-modulated when AML cells were cultured in presence of hBM-MSCs. The treatment of AML cells with Wnt/β-catenin inhibitors in addition to Ara-C or Idarubicine, cultured alone or in co-culture with hBM-MSCs, reduced AML cell proliferation and decreased AML cell viability. In the same conditions, GSK-3 inhibitors increased dramatically the AML cell apoptotic rate. *in vitro* observations were successfully translated into NOG mice, where LiCl in association with Ara-C significantly lowered leukemic engraftment in AML xenograft model, prolonging mouse survival as compared to Ara-C alone.

Conclusions: Wnt/β-catenin and GSK3 inhibitors reduce proliferation and chemoresistance of non-promyelocytic AML cells in co-culture with stromal cells. Furthermore, GSK3 inhibitors, such as Licl, improve the efficacy of some chemotherapeutic agents *in vivo* Wnt/β-catenin-GSK3 signaling may represent a potential therapeutic strategy to improve non-promyelocytic AML treatment.

PO071

IDENTIFICATION OF A NOVEL MUTATION PREDISPOSING TO FAMILIAL AML AND MDS SYNDROME BY A NGS APPROACH

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Introduction: Familial AML and MDS syndromes (FAMS), recently recognized in the WHO classification provide a useful model for investigation of predisposing genetic mutations. Genetic analysis of several pure familial leukemia pedigrees led to the discovery of well defined syndromes associated with inherited de novo mutations on germline DNA. Growing clinical awareness as well as a widespread use of NGS have led to an enlarged description of familial MDS/AML cases, and the number of mutations involved, suggesting they are more frequent than previously recognized. Despite the recent discovery of well-established causative gene mutations (RUNX1, GATA2, ETV6, TERT, TERC, SRP72, ANKRD26, DDX41, CEBPA), many cases remain unexplained (about 80%), suggesting that other inherited mutations could predispose to MDS/AML. New NGS approaches would help to the identification of more cases, genes, as well as novel syndromes.In 2017, we started a multicentric prospective study (Clinical trial.gov NCT03058588) aiming to look for predisposing mutations in patients and relatives affected by FAMS by NGS and to screen for old and new mutations potentially associated with the disease.

Methods: At present, 12 AML/MDS patients have been enrolled. Leukemic (bone marrow) and germline (buccal swab) DNA were analyzed by NGS gene panel approach based on a 28 genes associated to myeloid leukemogenesis, including the 9 above mentioned genes associated to FAMS. NGS libraries were performed by a Nimblegen (Roche) custom panel based on gene capture strategy and the sequencing was performed by MiSeq (Illumina).

Results: Ten patients did not reveal any germline mutations and the candidates are undergoing to whole exome sequencing. One presented a germline mutation on RUNX1, and the analysis of the affected relatives is on going. One revealed a new mutation. She was a 70 years old woman affected by RARS and her pedigree was characterized by 9 relatives affected by hematologic and solid neoplasia and trombocytopenia (Figure 1).The NGS analysis revealed the mutation c.*514C>T in 3'UTR of ETV6 with VAF of 50% on tumor DNA. The variant has never been described before, while ETV6 has been already associated with FAMS. Sanger sequencing confirmed the mutation on the germline DNA in heterozygosis. The screening of 2 affected relatives still alive confirmed the presence of the variant in heterozygosis. In silico analysis performed on PolymiRST Database revealed that c.*514C>T in 3'UTR of ETV6 results in a gain of miRNA binding site: hsa-miR-4717-3p and hsa-miR-942-3p.

Discussion: The variant c.*514C>T in 3'UTR of ETV6 seems to repress ETV6 due to RNA interference. The new binding miRNAs have been already described as over-expressed in solid and hematologic tumors. Moreover, the down-regulation of ETV6 is associated with alteration of cell growth and hematopoiesis. Due to these evidences, c.*514C>T in 3'UTR of ETV6 could be considered as a new mutation involved in FAMS predisposition.

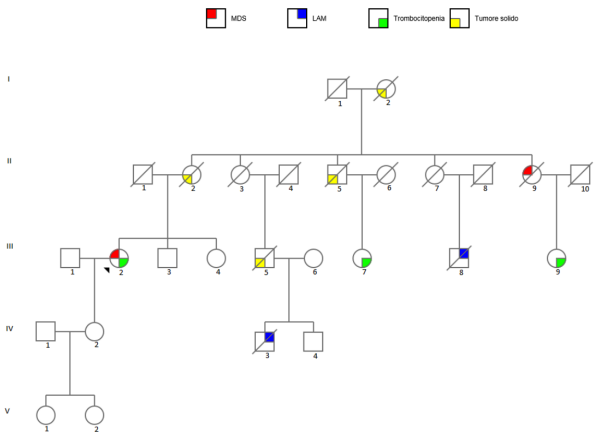

Figure 1.

PO072

TARGETING CELL CYCLE S-PHASE STABILITY BY THE CONCOMITANT INHIBITION OF CHK1 AND WEE1 KINASES IN ACUTE LYMPHOBLASTIC LEUKEMIA

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Background: Although several innovative therapies are today improving the outcome for adult ALL patients, a large percentage frequently relapse or became refractory to treatments. Thus, there is a need to improve the efficacy of actual treatments or to identify novel therapeutic strategy. The inhibition of cell cycle checkpoints has become a promising therapeutic option for the treatment of different tumors. The preclinical data available, led the basis for the clinical evaluation of this class of compounds. On ALL only few data on the efficacy of cell cycle checkpoint inhibitors has been reported. It has been showed that the simultaneous inhibition of Chk1 and Wee1 kinases synergizes in term of reduction of cell viability, induction of apoptosis and inhibition of proliferative capacity.

Aim: To evaluate the efficacy of the concomitant inhibition of Wee1 and Chk1 kinases in the treatment of ALL as single agent and in combination with the S-phase specific chemotherapy agent, methotrexate.

Methods: Gene expression analysis was performed using Affymetrix GeneChip Human Transcriptome Array 2.0 on leukemic cells isolated from the bone marrow and the peripheral blood of adult B-ALL patients. B-/T-ALL cell lines and primary cells were treated with PF-00477736 (Chk1 inhibitor) and/or AZD-1775 (Wee1 inhibitor) *in vitro*. The efficacy of the combination was evaluated in term of reduction of the cell viability, reduction of cell proliferation, cell cycle modification, induction of

apoptosis and protein modification.

Results: We showed that in primary B-ALL samples at diagnosis (n=39) WEE1 and CHEK1 transcripts are highly expressed and positively co-expressed (Pearson r = 0.5770, p = 0.0001). Having established that the abundance of WEE1 and CHEK1 is significantly high in ALL, we therefore next evaluated the efficacy of the concomitant inhibition of both kinases. The two small inhibitors PF-00477736 and AZD-1775 synergized in the reduction of cell viability (CI. between 0.17-0.9), induction of DNA damages, activation of cell apoptosis and increment of S phase cells in different ALL cell lines. Interestingly the combination compromised the viability also of primary ALL cells. To further evaluate the potential of the combination, ALL cell lines were treated with methotrexate after being exposed to PF-00477736 and/or AZD-1775. Interestingly the treatment with combination deeply sensitized different leukemic cell lines to the cytotoxicity of methotrexate. The specificity of the pharmacological schedule on S phase was confirmed using the G2/M phase specific inhibitor, doxorubicin, which showed no significant reduction of the cell viability.

Conclusions: We confirmed that the concomitant inhibition of WEE1 and CHK1 mine the viability of leukemic blasts inducing DNA damages and triggering apoptosis. We demonstrated that the inhibition of WEE1 and CHK1 kinases could be a winning strategy to enhance the toxicity of conventional chemotherapy.

PO073

CHK1 INHIBITION RESTORES INOTUZUMAB OZOGAMICIN CITOTOXICITY ON CD22-POSITIVE CELLS EXPRESSING MUTANT P53

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Introduction: Inotuzumab ozogamicin (IO) is an anti-CD22 calicheamicin immunoconjugate that has been recently approved for the treatment of relapsed or refractory B-Acute Lymphoblastic Leukemia (r/r B-ALL). We employed both immortalized and primary cells derived from CD22-positive lymphoproliferative disorders to investigate the signaling pathways contributing to IO sensitivity or resistance.

Methods: To calculate IC50 values, BL-2, Sup-B15, Namalwa and primary CD22-positive cells were exposed to logarithmic dilutions of IO. Multiple DNA-damage-induced proteins, cell cycle distribution and apoptosis were evaluated after exposure to IO, alone or in combination with the Chk1 inhibitor UCN-01. Immortalized cells bearing wild-type or mutant p53 were lentivirally transduced with mutant or wild-type p53, respectively.

Results: We found that the drug reduced the proliferation rate of CD22-positive cell lines expressing wild-type p53 (BL-2 and Sup-B15), but was less effective on cells exhibiting mutant p53 (Namalwa). In addition, cells surviving IO were mostly blocked in the G2/M phase of the cell cycle because of Chk1 activation resulting in induction of p21 in the presence of a wild-type p53 Background: However, when we combined IO with the Chk1 inhibitor UCN-01, we successfully abrogated IO-induced G2/M arrest regardless of p53 status indicating that the DNA damage response triggered by IO exposure is modulated by p53-independent mechanisms. The predictive value of p53 in determining IO responsiveness was also confirmed in primary CD22-positive cells derived from B-ALL patients at diagnosis and from patients with r/r B-ALL. Furthermore, co-treatment with IO and UCN-01 significantly increased cell death in primary cells expressing mutant p53.

Conclusions: Our findings suggest that p53 status may represent a biomarker predictive of IO efficacy in patients diagnosed with CD22-positive lymphoproliferative diseases.

PO074

8Q24/MYC REARRANGEMENT IN BLASTIC PLASMACYTOID DENDRITIC CELL NEOPLASM WITH T(6;8)(P21;Q24)

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Introduction: Blastic plasmacytoid dendritic cell neoplasm (BPDCN) is a rare hematological malignancy derived from the precursors of plasmacytoid dendritic cells. Patients with BPDCN have an aggressive clinical course and high frequency of cutaneous and bone marrow (BM) involvement. The neoplastic cells of BPDCN often express CD4, CD56, CD123, and TCL1. Most BPDCN show karyotypic abnormalities, often with complex karyotype with losses more common than gains of genetic material. Here, we reported a case of patient, a man of 69 years old, arrived at our hospital forsweating, pallory, asthenia and axillary lymphadenopathy. He exhibited the karyotype 46,XY,t(6;8)(p21;q24),der(13) with 8q24.1/MYC rearrangement, thus we hypothesize the possible involvement of gene SUPT3H at 6p21. This reciprocal chromosomal translocation, t(6;8)(p21;q24) was showed in four cases with BM infiltration and it appears to be a recurrent cytogenetic abnormality in BPDCN.

Methods: Laboratory data including white blood cell count, hemoglobin level, platelet count, percentage of blasts were collected. Flow cytometry immunophenotypic analysis was performed using a panel of antibodies designed for acute myeloid leukemia. Conventional chromosome analyses was performed on G-banded metaphase cells prepared from unstimulated bone marrow aspirate cultures for 24h. Fluorescence *in situ* hybridization (FISH) analysis with LSI D13S319/13q34 and LSI MYC break Apart probes was performed on harvested BM cells.

Results: The peripheral blood showed the white blood cell count of $21{,}31x10^3/\mu l$,a platelet counts of $82x103/\mu l$. Lymph node biopsy presented diffuse infiltration of atypical cells. Results of flow cytometry analysis revealed that tumor cells were positive for CD4, CD7,CD123,CD103 and CD123.On the basis of immunophenotypic findings, he was diagnosed as BPDCN. BM was highly infiltrated with tumor cells. Conventional cytogenetics analysis revealed: 46,XY,t(6;8)(p21;q24),der(13)[20] and FISH analysis demonstrated MYC rearrangement in 70% of cells and deletion of 13q34 in 90% of cells.

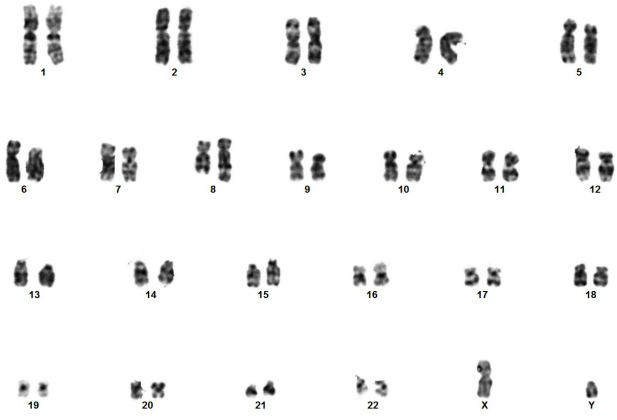

Figure 1. kARYOTYPE 46,XY,t(6;8)(p21;q24),der(13).

Conclusions: PDCN is often associated with a complex karyotype with genomic material losses. 8q24/MYC rearrangements occur in 10-15% of BPDCN, partnered with non-immunoglobulin chromosomal loci and may play a role in BPDCN pathogenesis. Because of small number of cases reported in literature, further investigation is needed to clarify the role of the chromosomal aberrations in BPDCN patients.

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PO075

TWO NOVEL DNMT3A MUTATIONS IN ACUTE MYELOID LEUKEMIA

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Introduction: Recurrent somatic mutations of DNMT3A occur in about 20% of AML patients. DNMT3A is a member of DNMTs family involved in DNA methylation. DNA methylation is an epigenetic mechanism regulating DNA accessibility and gene expression. In AML DNMT3A genomic alterations mostly consist in missense mutations targeting a hot spot site at R882 codon, that exhibit a dominant negative effect. R882H-R882C-R882P mutations of DNMT3A in primary AML samples are associated with a hypomethylated status of CpG islands (Suetake, J Biol Chem,2004).

Methods: Peripheral blood and/or bone marrow samples were collected from 180 AML patients during 2015-2016. DNA was extracted using Maxwell 16LEV Blood kit (Promega, Madison, Wisconsin, USA). Amplicon libraries were prepared by Myeloid Solution sequencing panel (SOPHiA GENETICS, Switzerland) starting from 200ng (Illumina) or 500ng (Ion Torrent S5) of DNA. Sequencing runs were performed on Illumina MiSeq and Ion Torrent S5 genome sequencers. Sequences obtained were mapped to human reference genome GRCh37/hg19 and annotated using SOPHiA DDM platform. DNMT3A mutations were validated by sanger sequencing. To detect DNMT3a, protein level was assessed by western blot after protein extraction using AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Marseille, France).

Results: We received in our laboratory 180 AML samples between 2015-2016, including 25 diagnosis, 16 relapse and 139 follow up. 11,1% AML patients showed DNMT3A mutations and 80% consisted in the canonical R882H or R882C amino acid substitution. We identified two new mutations in the DNMT3A gene by NGS, each of them was detected in one patient. Patient #1 had 70% of blasts and showed an undescribed single nucleotide variant of DNMT3A at exon 20 causing a premature STOP codon (cDNA c.2385G>A; tgG/tgA p.Trp795*; NM_022552;), copupled with IDH2 R172K (AGG/AAG) mutation. The DNMT3A mutation load was 40% in the diagnosis sample and drop down at 3% after Guadecitabine treatment (SGI-110-04) in the follow-up sample. Patient #2 carried an 80% of blasts with a new insertion of 36 aa in exon 22 of DNMT3A (c.2924_2925ins36;p.T862_E863ins12 i(263.5,TCATGAATGAGAAAGAGGACATCTTATGGTGCACT), along with FLT3-ITD. The DNMT3A mutation load was 30% at diagnosis and became undetectable at follow up, after chemotherapy treatment (FLAI-5). DNMT3A protein was undetectable in both mutated samples at diagnosis, with a reduction of trimethylation of lysine 36 of H3 histone (H3K36), which recruit DNMT3A on active enhancers. DNMT3A expression was restored in follow-up samples after patients' treatment.

Conclusions: We identified two new mutations in DNMT3A, which were not previously described in AML. These mutations show a dominant negative effect on Dnmt3a protein expression. We hypothesized that these mutations may alter protein or mRNA stability and increase their degradation rate. Further studies are needed to better understand the functional and pathological role of these alterations.

PO076

PRE-CLINICAL STUDIES ON PAX5 FUSION GENE TARGETING IN PEDIATRIC B-CELL PRECURSOR ACUTE LYMPHOBLASTIC LEUKEMIA

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Introduction: Despite the current risk-based stratification protocol, about 20% of pediatric patients with B-Cell Precursor Acute Lymphoblastic Leukemia (BCP-ALL) experience relapse. Ph-like (or BRC/ABL-like) is a novel high risk subgroup, which represents 15% of BCP-ALL patients. The transcriptional factor PAX5 is frequently involved in several translocations in Ph-like patients, determining the formation of fusion genes encoding for aberrant proteins. This project aims to understand the role of PAX5 fusion genes in leukemogenesis, elucidating the involved signaling pathways, in order to develop new strategies to strike the disease. Moreover, it aims to evaluate the efficacy of new specific tyrosine kinase inhibitors, such as Nintedanib/BIBF1120.

Methods: We setup *ex vivo* co-culture of Human Bone Marrow Stroma cells (HBMS cell line) and primary leukemic blast of patients. We performed drug assays with Nintedanib, alone or in combination with conventional chemotherapy agents, assessing cell viability by Annexin V assay (FACS analysis). Furthermore, we setup an in-vivo model of xenotransplantation of human leukemic cells, carrying PAX5 fusion genes, into NSG mice. In this model, we investigated the efficacy of Nintedanib to target signaling and viability in tumor cells. In particular, we realized phosphoflow (FACS analysis) to study signaling in vehicle vs. treated mice.

Results: Our preliminary studies showed that PAX5 fusion genes sustain survival of leukemic cells leading to hyper-activation of LCK. *Ex vivo* treatments with Nintedanib on primary BCP-ALL samples demonstrated its efficacy both in monotherapy and in combination with standard chemotherapy, such as Dexamethasone, Asparaginase or Vincristine. We obtained comparable results in all patients, characterized by different fusion genes: PAX5/AUTS2; PAX5/DACH2; PAX5/SOX5 and PAX5/JAK2. *In vivo* experiments, further confirmed ex-vivo data,especially in bulk disease setting (high level engraftment). In addition, the combination with Dexamethasone synergized the treatment efficacy. Finally, Phosphoflow analysis in treated samples showed the involvement of Akt pathway (FACS).

Conclusions: Overall, this study proposed PAX5 fusion proteins as a good target for novel approaches in BCP-ALL. Both *ex vivo* and *in vivo* results demonstrated the efficacy of Nintedanib on cases carrying PAX5 fusion genes,acting both on LCK and Akt pathway, with significant effects also in monotherapy. Furthermore, these data suggested use of Nintedanib to improve the outcome of this specific subgroup of patients. Moreover, in combination with standard chemotherapy, it may lead to a decrease in the dosage of the latter. Consequently, combination treatments can guarantee a reduction in the toxicity of the treatment, a critical aspect especially in pediatric patients.

PO077

HIGH RECURRENCE OF FUSION GENES IN PHILADELPHIA-LIKE CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

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Introduction: Ph-like (or BCR/ABL-like) ALL subtype encompasses

10-15% of BCP-ALL patients, predicts high incidence of relapses and defines a candidate subgroup for targeted treatment. The present study, aims (i) To identify BCP-ALL Ph-like cases in patients treated in Study Protocols of the Italian Association of Pediatric Hematology and Oncology (AIEOP); (ii) to assess their prognosis; and (iii) to characterize their genetics basis, in terms of CNV and fusion genes.

Methods: Gene expression profiling was successfully performed on 400 Italian childhood BCP-ALL cases enrolled in AIEOP-BFM ALL2000/R2006 protocols. Of them, 138 negative for common fusion transcripts, non-high hyperdiploid and non-Down Syndrome, were defined as B-others. RNA-target-NGS (panel with 1385 cancer-associated genes, Trusight RNA Pan Cancer, Illumina) has been setup to identify fusion genes involving recurrent genes with novel partners.

Results: Out of 138 B-other cases, 59 (43% of B-others, and 15% of the total BCP-ALL cohort) presented as a cluster with a gene expression signature close to the BCR/ABL signature, therefore referred to as Ph-like. Among B-others, Ph-like cases had a significantly increased proportion of males, age>10 years and WBC>20x109/L. Overall, 33 out of 138 cases experienced relapse, 20 Ph-like (34%) and 13 B-others not Ph-like (16%). We analyzed 134/138 B-others by MLPA and detected 11/138 IKZF-plus patients (9/11 Ph-like and 6 experienced relapse). By NGS, we analyzed 72/138 patients, including all the Ph-like cases plus relapsed cases B-other not Ph-like. Among the 20 Ph-like relapsed cases, 13 were positive for a fusion gene, while the three out of seven cases negative were IKZF-plus and the remaining, two had deletion in BTG1 and two were normal karyotype. Overall, among Ph-like, we detected fusion genes with recurrent partner genes, such as nine cases carrying P2RY8/CLRF2, 7 with PAX5 fusion genes, 3 with EBF1-fusions, 1 case with TCF3/HLF, 1 with IKZF1-fusion, and 1 case with BCL9/MEF2D, plus additional single cases with novel fusion genes. We further identified IgH-CRLF2 (FISH) in two cases, one with a concomitant PAX5-fusion. Overall, 35/59 Ph-like cases were carrying a relevant fusion gene. Among relapsed B-others not Ph-like patients, one carried ZNF384/CREBBP fusion gene, originated from translocation t(12;16) and one was positive for the atypical t(12;21), not identified by conventional RT-PCR screening.

Conclusions: We dissected the Ph-like subgroup in the Italian cohort of children with BCP-ALL, showing that, independently of other known risk features, these patients have a poor outcome and can be considered eligible for alternative treatments, in particular when they have high levels of MRD after Induction. The characterization of this subgroup revealed a high recurrence of fusion genes, this finding underlining the urgent need to define the pathogenetic mechanisms, with the aim to identify novel and targeted therapies.

PO078

VALIDATION OF MULTI-TARGET NEXT GENERATION SEQUENCING PANEL FOR THE GENETIC CLASSIFICATION OF ACUTE MYELOID LEUKEMIA: A SINGLE CENTRE EXPERIENCE

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Introduction: AML genome is one of the simplest cancer genomes, 20 genes are significantly mutated and an exhaustive molecular characterization performed by standard technique is quite time consuming; for this reason the use of targeted next-generation sequencing (NGS) is no more delayed. We planned to perform a internal validation program of targeted NGS strategy with two different commercial NGS workflow (NGSW1 and NGSW2).

Methods: NGS panels were composed from 30 genes (hotspot and full genes). 33 AML samples, previously characterized by conventional assay and Sanger sequencing for mutational status of FLT3, NPM1, WT1, IDH1, IDH2, CEBPA, DNMT3A, ASXL1 and RUNX1, were used as a training and validation set to setup two different commercial NGS workflow (W1 and W2). Training set (12 samples for W1 and 8 samples for W2) was useful to fit analysis filters to improve variant caller and reduce false positive and false negative. After training set, 13 new samples were used as Validation set to verify NGS workflow performance. For both NGS workflow we performed two experiments according to the manufacturer's instructions: libraries preparation, sequencing and bioinformatic pipeline. NGS W1 library preparation is an amplicon-based strategy and W2 is a capture one.

Results: NGS experiments showed a good coverage, with minimal coverage not less than 500x in about 95% of regions for W1(mean coverage 1087) and in 100% for W2 (mean coverage 4494); also showing good reads distribution between samples (Uniformity>95%, W1 mean 690.000 and W2 2.800.000 reads). In the training set W1 did not identified 5 indel variants (false negative, FN) out of 21 total somatic mutations showing a sensitivity of 76%, the W2 showed 2 FN indel variants out of 13 somatic mutations with 85% sensitivity, globally 5/7 FN interest homopolymer region. These results required a change in the filtering parameters. Both NGS workflow showed a specificity of 100%. In validation set, albeit samples number needs to be incremented, almost all mutations except one were correctly identified by both workflow obtaining about 98% of sensitivity; however the FN variant was detected by integrative genomics viewer (IGV) visual alignment review. Our experience suggest that the presence of large in/del and homopolymer regions may be investigate by IGV visualization.

Conclusions: Amplicon-based library preparations require much smaller quantities of input DNA and the workflow tends to be simpler and less time and labor-intensive compared to capture-based methodologies but is less performing in sequencing of GC rich and homopolymer regions. Hybridization capture-based approaches demonstrate better uniformity of coverage and generate fewer false-negative but protocol is more labor-intensive, with different critical phases needing high manual skills. Assessment of a exhaustive molecular by NGS technique may be very helpful to characterize different subcategories of AML but advice in the interpretation of NGS data may due take in account. This work was supported by a grant of Assessorato alla Salute Regione Sicilia (PSN 2016)

PO079

OFF LABEL USE OF 10-DAYS DECITABINE SCHEME IN A SMALL SERIES OF UNDER 61-YRS OLD SECONDARY AML WITH TP53 MUTATION: ANCONA EXPERIENCE

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Decitabine is actually approved in AML patients ineligible to intensive chemotherapy with a 5 days scheme of infusion. Welch *et al.* administered Decitabine for 10 days in the very unfavorable setting of TP53 mutated AML achieving a superior number of CR in comparison to historical controls reported by the literature (100% vs 20-40%) with an intriguing median OS of 12 months and the abolition of the detrimental effect of this mutation after Allogeneic Transplant. We therefore decided to enroll four secondary AML patients aged 52-60 yrs old in a 10-days Decitabine (20 mg/sm/d) off label protocol, approved by our ethical committee between 2017 and 2018. All patients received Quinolone and Posaconazole prophylaxis and were admitted to hospital during treatment and infectious complications, but after achieving CR further courses were reduced at 5 days and administered outpatient. The first 53 yrs old patient was affected by AML secondary to myelofibrosis, with del17p and TP53 mutation. She received 4 Decitabine 10-days courses complicated by FUO after the second and the third cycle. She achieved a stable disease on the basis of PB blasts counts, since bone marrow aspirate was not evaluable and she died of sepsis after the last treatment. She overall survived 5 months with erytroid transfusion dependence. The second 52 yrs AML patient had a therapy related disease with complex Karyotype, TP53 mutation and received 4 Decitabine courses, the first reduced at 5 days to explore tolerance in a patient heavily pretreated for Hodgkin Disease and sarcoma of the bone. She was refractory and the treatment was complicated by a FUO and a pneumonitis after the third and the fourth

course. She obtained CR with negative MRD after salvage treatment with Venetoclax, but she died during Transplant Conditioning of Escherichia Coli sepsis. She overall survived 8 months. The third 60-yrs old patient affected by complex Karyotype secondary AML with TP53 mutation received 2 10-days course and 6 5-days courses after achieving CRi with platelet and RBC transfusion dependence, but a negative MRD (WT1 and MPFC). She died of severe gut aGVHD in relapse after 8 months of treatment. The last 60 yrs secondary AML patients with complex karyotype and TP53 mutation achieved CRi with transfusion independence after the 2nd course, she received Allogeneic Transplant from haploidentical donor in December 2017, after 5 courses and is still alive in CCR with negative MRD (WT1 and MPFC) after 11 months of treatment. In conclusion 50% of patients responded to this off label protocol with one patient achieving MRD negativity in a very unfavorable setting. We are planning to study mutational clearance of TP53 in the 2 responding patients using stored frozen bone marrow samples. Further studies should explore feasibility and efficacy of these scheme in TP53 mutated AML usually charactherized by dismal prognosis even after Allogeneic Transplant.

Chronic Lymphocytic Leukemia and Chronic Lymphoproliferative Disorders 2

PO080

CD200 INCLUDED IN A 4-MARKER MODIFIED MATUTES SCORE PROVIDES OPTIMAL SENSITIVITYAND SPECIFICITY FOR THE DIAGNOSIS OF CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction: Mature B-cell leukemias are heterogenous disorders currently included in the "mature B-cell lymphoid neoplasms". Despite significant progresses in cytogenetics and molecular biology, morphological cell identification and immnologial marker analysis by means of flow cytometry remain cornerstones in distinguishing between the different entities and difficulties in definining some cases still exist. CD200, a transmembrane type Ia glycoprotein belonging to the immunoglobulin superfamily, has been shown to have a differential expression in B cell neoplasms, thus appearing useful in differential diagnosis.

Methods: Here we retrospectively assessed the diagnostic relevance of CD200 on 427 patients with B-cell chronic lymphoid leukemias (median age 69 years; range 35-97 years) seen at our Institutions between June 2008 and August 2017.

Results: The median age of patients at diagnosis was 69 years (range 35-97 years), 282 were male (66%) and 145 were female (34%), with a M:F ratio of 1.9. The final diagnosis based on the investigator's assessment was chronic lymphocytic leukemia (CLL) in 75% of cases and non-CLL in the remaining 25% of cases: 53 marginal zone lymphomas, 21 mantle cell lymphomas, 15 hairy cell leukemias, 1 follicular lymphomas, and 4 lymphoplasmacytic lymphomas. Sensitivity and specificity for the diagnosis of CLL (vs non-CLL) were calculated for the following markers: CD200, CD5, CD22, CD23, CD79b, FMC7, and SmIg. CD23 was the only marker without a statistically significant difference between the investigator assessment and the flow cytometric analysis. The other markers were unable – when individually evaluated - to discriminate between CLL and non-CLL, requiring the integration into a scoring system. The modified score #1 (addition of CD200) showed superimposable sensitivity and specificity compared to the Matutes score. The substitution of CD79b (modified score #2), surface membrane immunoglobulins (SmIg) (modified score #3), CD79b and FMC7 (modified score #4) with CD200 showed that only the modified score #4 had both higher sensitivity and higher specificity compared to standard Matutes score.

Conclusions. This work, based on a very large cohort of patients, defines a simplified score, compared to the classical Matutes score, for the differential diagnosis of chronic B-cell leukemias – which only requires 4 markers instead of 5 (CD5, CD23, CD200, SmIg). Certainly, this finding needs further confirmation by means of an external validation, before being proposed as routine diagnostic procedure.

PO081

INTRACELLULAR CALCIUM MOBILIZATION MEDIATES FOCAL ADHESION KINASE (FAK) ACTIVATION IN POOR PROGNOSIS CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS

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Introduction: Signaling events downstream the B Cell Receptor (BCR) engagement are central for the progression of B cells from Chronic Lymphocytic Leukemia (CLL). Focal adhesion kinase (FAK) is the primary enzyme involved in the engagement of integrins and assembly of focal adhesions thus playing a major role in cellular adhesion and metastasis of various cancers. FAK is regulated by Calcium (Ca2+) flux and by Src family kinases (e.g. Lyn) through a Calpain-dependent manner. FAK has been demonstrated to be over-expressed in many human cancers but a down-modulation of its expression has also been reported. The aim of this study was to investigate and characterize FAK protein in CLL patients.

Methods: FAK expression was analyzed in B-lymphocytes from 100 CLL patients and 10 healthy subjects by Western blotting (WB). In 20 patients, surface IgM and IgD expression was evaluated by flow cytometry (FC). For Ca2+ mobilization assessment, 1x10^7 cells were incubated with 4μM Fluo-4-AM at 37°C for 30min and then analyzed by FC; after 30s of baseline acquisition, α-IgM F(ab')2 and α-IgD F(ab')2 (10 μg/ml) were added and fluorescence intensity was recorded for 5 min. Ionomycin was added as positive control. 50μM PD150606 was used to inhibit Calpain.

Results: WB analyses revealed a slightly significant difference in FAK expression between patients and controls ($p<0.05$) being the protein down-regulated in the first as compared to the latter. When we correlated FAK expression with prognostic indexes (i.e. IGHV somatic hypermutations or cytogenetic lesions), we found a significant decrease of FAK in poor-prognosis patients. To a more careful study, we observed that FAK down-regulation was limited to its whole form detected in WB at 125kDa, while bands related to FAK cleavage (92/94kDa) were detected also in those patients lacking full length-FAK. Cleaved-FAK is due to Calpain proteasic activity, since Calpain inhibition avoids FAK cleavage. Considering that Calpain activation is mediated by Ca^{2+} flux, we compared FAK expression to IgM/IgD surface expression and the capability of the cell to respond to BCR stimulation in term of Ca^{2+} mobilization. This analysis highlighted that patients with IgM or IgD expression and Ca^{2+} flux have less amount of full length-FAK, which translates into a higher presence of cleaved/activated form of FAK.

Conclusions: We herein propose that full length-FAK down-modulation could be considered as a new marker of unfavorable prognosis. In this model, poor prognosis CLL patients presenting IgM and/or IgD and Ca^{2+} mobilization, are more prone to activate Calpain, which in turn activates FAK. Together with data from literature, our results suggest that CLL cells missing the full length-FAK, not only are unaffected by the lack of it, but rather they present an activated form of FAK that could favor cell migration and metastatic invasion.

PO082

BORTEZOMIB DECREASES IL-6 PROTEIN LEVELS IN T-LARGE GRANULAR LYMPHOCYTE LEUKEMIA LEADING TO MONOCYTES DEPLETION

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Introduction: T-Large Granular Lymphocyte (T-LGL) Leukemia (T-LGLL) is a chronic lymphoproliferative disorder characterized by the clonal expansion of CD3+CD8+CD57+ T-LGLs. By inducing the activation of pro-survival signaling pathways, e.g. JAK/STAT, in leukemic LGLs, pro-inflammatory micro-environment plays a central role in T-LGLL pathogenesis. The major activator of JAK/STAT axis is IL-6, which was found at higher levels in plasma of T-LGLL patients than controls, mainly released by LGLs-depleted PBMCs. The expression of several pro-inflammatory cytokines, as IL-6, is mainly regulated by NF-kB transcription factor and the proteasome inhibitor Bortezomib (Bz) has been reported to down-regulate NF-kB activity by preventing the proteasomal degradation of its inhibitors, IkBs proteins. Provided its potential anti-inflammatory role, Bz has recently been proposed in clinic for inflammatory conditions, as Rheumatoid Arthritis, an autoimmune disorder often associated with T-LGLL. In order to identify the population that sustains T-LGLs survival, this work aims to study IL-6 production and to evaluate the anti-inflammatory properties of Bz in T-LGLL.

Methods: CD14+ cells were purified from PBMCs of T-LGLL patients by magnetic micro-beads/columns system. Transcriptional and protein expression levels were evaluated by Real Time PCR, Western Blot (WB) assays and ELISA test. Cell apoptosis was evaluated by AnnexinV staining and Flow Cytometry (FC).

Results: To investigate cells putative responsible for IL-6 production, we purified CD14+ monocytes from PBMCs of patients, demonstrating that IL-6 transcriptional levels were significantly higher in these cells as compared to the CD14- fraction. To evaluate Bz anti-inflammatory properties, patients' PBMCs were cultured with Bz 5nM and IL-6 transcription was analyzed after 24h. Thus, a 64% IL-6 decrease was demonstrated in treated PBMCs as compared to the control condition; data were also confirmed at protein levels by ELISA test. Moreover, we provided evidence that this result was related to the significant reduction of monocytes detected by FC after 24h of Bz-treatment as compared to the untreated condition. Then, by WB assays, we investigated Bz effects on JAK/STAT axis activation, evaluating STAT3 phosphorylation at Y705 (pSTAT3 Y705). Our data indicated that a pSTAT3 Y705 decrease in Bz-treated PBMCs was detectable soon after 24h, though the major difference was observed after 48h. Finally, we assessed cell viability after 24-48h of Bz-treatment, showing a significant time-dependent increase in CD57+ LGLs apoptosis as compared to controls.

Conclusions: We herein provide evidence that monocytes are the major producers of IL-6 in T-LGLL. We also demonstrated the anti-inflammatory role of Bz in reducing IL-6, through monocytes depletion. Thus, we point to an indirect mechanism of action of this drug, leading to T-LGLs apoptosis by reducing IL-6-mediated activation of the pro-survival JAK/STAT axis.

PO083

CCL5/IL-6 LOOP SUSTAINS PRO-INFLAMMATORY MICROENVIROMENT IN A SUBSET OF T-LARGE GRANULAR LYMPHOCYTE LEUKEMIA PATIENTS

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Introduction: T-cell large granular lymphocyte leukemia (T-LGLL) is a rare lymphoproliferative disorder characterized by the clonal expansion of T large granular lymphocytes (LGLs). The survival of the leukemic clone is mediated either by activating mutation in key genes, as STAT3, or by a chronic pro-inflammatory stimulation. Our group has demonstrated that IL-6 and the soluble form of its specific receptor, IL-6Rα, were present at higher concentration in patients' plasma than healthy controls and they were produced by the non-leukemic fraction of the peripheral blood mononuclear cells. IL-6 was also demonstrated to induce STAT3 phosphorilation in T-LGLL patients. Other cytokines and chemokines were identified more present in patients' plasma, such as CCL5. Anyway, a low expression of CCR5, one of CCL5 receptors, was demonstrated on LGLs surface. Therefore, in order to better understand

the mechanisms involved in LGLs survival, we pointed to better characterize the cell source of CCL5 and IL-6 in T-LGLL patients and to identify the putative relationships between these two cytokines in the pathogenesis of T LGLL.

Methods: The study included 15 patients with T-LGLL, and 5 healthy controls. Immuno-magnetical purification was performed to obtain monocytes or LGLs. The expression level of CCL5, IL-6, IL-6Rα and CCR5 was measured by RT-qPCR. LGLs and monocytes were stimulated by IL-6 (20 ng/ml) or CCL5 (100 ng/ml).

Results: We pinpointed that CCL5 was selectively produced by LGLs and its expression was higher as compared to healthy controls. We subsequently identified that the source of IL-6 was represented by monocytes, distinguishing two groups of patients: one expressing significant higher levels (IL-6 high) and one with comparable amounts (IL-6 low) in respect to controls. We then identified that in the IL-6 high group, IL-6Rα and CCR5 were more expressed in comparison to the other two groups. After treating monocytes with CCL5, we showed that the expression of IL-6, mediated by CCL5, was higher in the IL-6 high group in respect to the other two. Treating LGLs with IL-6, we identified that IL-6 mediated CCL5 production was equally induced in LGLs obtained from the two groups of patients, although at a higher extent as compared to healthy controls.

Conclusions: These data demonstrated that the main source of IL-6 was represented by monocytes, even though in a subset of T-LGLL patients. The correlation between higher expression of IL-6, IL-6Rα and CCR5 in patients' monocytes, and the increased production of CCL5 by LGLs, suggested a possible cross-talk between LGLs and monocytes in these patients, through a CCL5/IL-6 loop. We demonstrated that this loop contributed to sustain the pro-inflammatory micro-environment particularly in the IL-6 high group of T-LGLL patients, playing a role in sustaining the LGLs survival. Studies are in progress to investigate the role of other cytokines, namely IL-15, on the inflammatory background of T-LGLL patients.

PO084

E3 UBIQUITIN LIGASE C-CBL AND ITS INTERACTION WITH BCR MOLECULES: STUDY OF CELL HOMEOSTASIS REGULATION IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction: Abnormalities of molecules involved in B-cell receptor (BCR) down-regulation have been linked to the pathogenesis of Chronic Lymphocytic Leukemia (CLL). Ubiquitylation of specific receptor tyrosine kinases by the E3 ubiquitin ligase c-Cbl, such as Lyn, provides a sorting signal for lysosomal degradation and leads to termination of receptor signaling. Recently, in colorectal cancer (CRC) Cortactin overexpression correlates with inhibition of ubiquitin-mediated degradation of EFGR by suppressing the coupling of c-Cbl with EGFR. We previously demonstrated that Cortactin, a Lyn substrate which couples the endocytic machinery to dynamic actin networks, is overexpressed in CLL patients with bad prognosis and this overexpression contributes to tumor cell invasion and metastasis. In order to elucidate the mecchansims that regulate the down-regulation of BCR signaling, we here investigated the expression of c-Cbl and its interaction to Cortactin.

Methods: Blood samples were collected from 15 controls and 30 CLL patients. Untouched peripheral blood B cells were purified using the RosetteSep isolation kit for human B cells. We characterized the protein level of Cortactin and c-Cbl by Western blotting and Flow-Cytometry. To evaluate the interaction between c-Cbl to Cortactin and Lyn in CLL B cells we performed a co-immunoprecipitation assay, followed by Western blotting analysis. Lyn and c-CBl ubiquitination was evaluated at steady state and after IgM (10 μg/ml) stimulus.

Results: We highlighted that in CLL B cells both c-Cbl and Lyn were overexpressed and there was a positive correlation between expression levels of two proteins was shown. However, we demonstrated that Lyn and c-Cbl did not associate nor ubiquitinated upon BCR engagement. Moreover, we detected that in CLL patients with Cortactin overexpression, c-Cbl and Cortactin were constitutively associated while in patients with lower Cortactin expression the two proteins did not co-immunoprecipitate, independently from c-Cbl protein level.

Conclusions: In CLL cells the absence of a relationship of c-Cbl with Lyn, also after BCR triggering, together with the association to Cortactin in those patients in which Cortactin is highly expressed, support the hypothesis that c-Cbl has adaptor functions that influence its activity, and in turn cell homeostasis.

PO085

REGULATION OF HIF-1 α IN TP53 DISRUPTED CHRONIC LYMPHOCYTIC LEUKEMIA CELLS AND ITS POTENTIAL ROLE AS A THERAPEUTIC TARGET

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Treatment of high-risk chronic lymphocytic leukemia (CLL) patients remains an unmet clinical need. Disease aggressiveness can be ascribed to intrinsic features of the tumor cells (i.e. TP53 disruption) and to the interaction of CLL cells with stromal cells (SC) of the microenvironment. HIF-1α is a transcription factor implicated in cell adaptation to hypoxia and is involved in the regulation of genes implicated in tumor progression. In CLL cells, HIF-1α is constitutively expressed even in normoxia and regulates the protective interactions that the leukemic cells establish with the microenvironment. The aims of this study were to understand HIF-1α regulatory pathways in CLL cells from TP53 disrupted and wild type patients, and to explore its role as a therapeutic target. Del(17p) in CLL cells was assessed by FISH and the presence of TP53 mutation was evaluated by sequencing. CLL patients with TP53 mutation, or >40% del(17p) were included in the TP53dis subset. Patients with <10% del(17p) and without TP53 mutation were considered TP53wt. CLL cells were cultured with or without M2-10B4 SC, and exposed to PD98059, Y27632, LY249002, BAY87-2243, F-ara-A or ibrutinib. Ras, ERK1-2, Akt, HIF-1α, Elk3 and pVHL expression was evaluated by Western Blot. RhoA and RhoA kinase acitivity was measured by specific immunoassays. HIF-1A, p21 and ENO1 gene expression was assessed by RT-PCR. Cell viability was analyzed by AnnexinV/propidium Iodide assay. We found that CLL cells from patients carrying TP53 abnormalities (TP53dis CLL cells) had higher transcriptional activity and expression levels of the α subunit of HIF-1 compared to CLL cells from TP53wt samples (TP53wt CLL cells). HIF-1α upregulation detected in the TP53dis subset was due to a reduced expression of the HIF-1α ubiquitin ligase pVHL and more active PI3K/Akt and Ras/ERK1-2 signalling pathways. Hypoxia and SC further enhanced HIF-1α accumulation in both TP53dis and

TP53wt CLL cells. Hypoxia-mediated HIF-1α upregulation was due to a decreased pVHL expression and to the activation of PI3K/Akt and Ras/ERK1-2 signalling pathways. SC did not affect pVHL expression, but induced an increased activity of Ras/ERK1-2, RhoA/RhoA kinase and PI3K/Akt pathways, leading to HIF-1α accumulation. *in vitro* fludarabine-resistant CLL cells were mostly TP53dis and expressed significantly higher levels of HIF-1A mRNA compared to fludarabine-sensitive cells. The HIF-1α inhibitor BAY87-2243 reversed the constitutive fludarabine resistance of TP53dis CLL cells, and counteracted the fludarabine resistance induced by SC. BAY87-2243 also elicited a strongly synergistic cytotoxic effect in combination with ibrutinib. Overall, our data indicate that HIF-1α is overexpressed in CLL cells, especially in the presence of TP53 abnormalities, and is susceptible of positive regulation by hypoxia and SC. HIF-1α can be regarded as a crucial target whose inhibition warrants further evaluation, also in combination with currently available therapies.

PO086

DESIGN AND MINION TESTING OF A NANOPORE SEQUENCING SPECIFIC GENE PANEL FOR CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction: MinION is a single-molecule nanopore sequencer from Oxford Nanopore Technologies connected to a laptop through a USB 3.0 interface. Sequencing is performed by the moving of individual DNA strands through biologic nanopores on a chip, where an electric field is applied and electrical signal variations are recorded. In the last years, next generation sequencing (NGS) methods have identified a wide range of gene mutations which have improved our knowledge about chronic lymphocytic leukemia (CLL), allowing to refine both the prognostic subgroups and better therapeutic strategies. In this study, we report a custom gene panel assay based on multiplex long-PCR followed by sequencing on MinION to identify single nucleotide variations (SNV) and insertions/deletions (indels) in 5 prognostically relevant genes in CLL: TP53, NOTCH1, BIRC3, SF3B1 and MYD88.

Methods: We designed a custom gene panel consisting of 7 primers pairs in 2 pools, with a total panel size of 15kb. Twelve patients were selected according to specific cytogenetic and molecular features significantly associated with the mutational status of these genes. For each DNA sample, 2 multiplex long-PCRs were prepared. MinION library preparation, sequencing and data analysis were performed. All cases included in the study were analyzed by Sanger Sequencing (SS) or other molecular assays for all the targets included in the gene panel, in blinded manner.

Results: Read depth analysis showed that the range of sequencing depth was inversely related to the amplicon size, with the smaller amplicons having a higher coverage (up to 2100x); the minimum read depth was never below 50x. The error rate calculated was on average 6% and 2% for indels and SNV, respectively. Except for the known mutation hotspots of NOTCH1 and MYD88 and the polymorphisms identified, considering the low chance to find a rare variant simultaneously in a small cohort, the variants occurring in multiple samples were excluded from further validation analyses; consequently, the actual coverage of the custom panel was 94,7%. These data are closely related to the chemistry and basecalling algorithms used and are probably intended to improve with the progress of nanopore technology. Overall, 8 pathogenic mutations were detected in 6 patients, with 2 patients harboring concurrently 2 mutations: 6 SNV and 2 indels. The lowest mutation allelic ratio was around 10%. These mutations were simultaneously identified and confirmed with SS or other molecular assays.

Conclusions: This is the first report of targeted sequencing based on a custom panel of pre-pooled multiplexed primers on MinION. This approach offers a rapid, easy and affordable workflow of analysis compared to SS or the common NGS platforms, even if it is still not ready to substitute the other NGS platforms because of MinION error proneness. Anyway, the rapid and constant improvements of nanopore technology promise an exclusive and convenient use of MinION in the future.

PO087

SELINEXOR (KPT-330) IN COMBINATION WITH CHEMOTHERAPY OR IDELALISIB ELICITS A SYNERGISTIC CYTOTOXIC EFFECT IN PRIMARY CLL CELLS, ALSO OVERCOMING INTRINSIC AND STROMAL CELLS-MEDIATED FLUDARABINE RESISTANCE

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Background: Despite the therapeutic efficacy of new target drugs in chronic lymphocytic leukemia (CLL), treatment of high-risk patients remains an unmet clinical need. Tumor suppressor and growth regulatory proteins with a pathogenic role in CLL bind the nuclear export protein exportin-1 (XPO1) and are carried through the nuclear pore complex into the cell cytoplasm. Elevated protein levels of XPO1, and mutations have been reported in hematologic and solid tumors. In particular, XPO1 is overexpressed and recurrently mutated in CLL cells. Selinexor (KPT-330) an oral inhibitor of XPO1, is active as single agent in different hematologic malignancies.

Aim: The aim of this study is to evaluate the additive or synergistic *in vitro* cytotoxic effects of selinexor, used in combination with chemotherapeutic drugs or the PI3k inhibitor idelalisib against primary CLL cells. Specifically, this study aims at identifying combination regiments that might overcome single agent resistance.

Methods: Purified CLL cells from 30 patients were exposed, alone or in presence of the murine stromal cell line, to selinexor (Sel) in combination with fludarabine (F-ara-A), bendamustine (Ben) or idelalisib (Ide) for 72 hours. Cell viability was analysed by Annexin-V/propidium Iodide immunostaining and flow cytometry. Combination analysis was performed using Calcusyn software. NF-kB protein amount was detected by western-blot.

Results: Leukemic cells were cultured in the presence of Sel, used alone or in combination with F-ara-A, Ben and Ide. After 72 hours of culture, the percentage of viable cells significantly decreased after dual treatment with Sel (100 nM) and F-ara-A (1 uM) compared to single agents and untreated controls. Combination analysis showed that Sel and F-ara-A synergize to induce CLL cells apoptosis with a CI < 1. Similarly we observed a synergistic interaction between Sel (100 nM) and Ben (30 mM) that significantly enhanced the cytotoxic effect of each individual drug with a CI <1. The combination of Sel (100 nM) and Ide (10 nM) at 72 hours resulted in a weaker, although significant, viability reduction. From the molecular standpoint, the combination of Sel and Ide caused a reduction in NF-kB total expression and activity in CLL cells. We observed that IGHV unmutated (UM) CLL cells showed high reduction of cell viability when exposed to synergistic combinations, compared to IGHV M cells. Sel was also effective in impairing the viability of CLL cells that showed intrinsic resistance to F-ara-A. Lastly, we exposed CLL-stromal cells co-cultures to the identified synergistic combinations, and found that all dual treatments significantly reduced the viability of leukemic cells, counteracting the protective effect exerted by stromal cells toward drug-induced apoptosis.

Conclusions: Our data demonstrate that the combination of Sel with chemotherapy or Ide has synergistic cytotoxic effects, also counteracting intrinsic or stromal cells-mediated drug resistance.

P0088

PROGNOSTIC VALUE OF CYTOGENETIC ABERRATIONS AND INTRATUMORAL GENETIC HETEROGENEITY IN CHRONIC LYMPHOCYTIC LEUKEMIA (CLL)

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Background: On the basis of current Dohner hierarchical classification, FISH analysis is still considered the gold standard for detection of recurrent cytogenetics aberrations (abs) with prognostic value in CLL patients (pts) [del(13q): favourable, +12: neutral, del(11q) and/or del(17p): adverse]. However, prognosis remains heterogeneous in each cytogenetic group and Intratumoral Genetic Heterogeneity (ITH), or the coexistence of different subclones, may contribute to this non uniformity. Here we aimed to validate a novel prognostic algorithm proposed by Yi et al., that integrates the number of recurrent cytogenetics abs and the size of intratumoral genetic subclones. This model allows to include in the favourable group also high risk cytogenetics abs (deletion of TP53 and ATM) when present as minor clone and, on the other hand, in the unfavourable group three concomitant neutral abs [del(13q),+12, t(14q32)]. To get insights into clonal composition, we performed an in-depth molecular cytogenetic analysis by two-colour probe sets.

Methods: PB samples were collected from unselected 58 CLL pts at diagnosis and/or before starting treatment. After short term cultures with CpG-oligonucleoitide DSP30/IL-2 and TPA, FISH was performed with a standard panel probes: XL DLEU/LAMP/12CEN and XL ATM/TP53 (cut-off: 10%). We distinguished a major or minor clone if the difference between 2 coexistent abs in each patient was >30%. Pts were then stratified in 3 groups (favourable, neutral, adverse) on the basis of the current and novel prognostic model. OS was assessed by Kaplan Mayer method and the prognostic features by long-rank test (P<0.05), with a median follow-up of 2yrs.

Results: FISH detected abs in 49 pts (84%) as following: a single aberration in 28 (57%), 2 abs in 14 (29%), 3 abs in 5 (10%) and 4 abs in 3 (6%). Basing on these data, in combination with the clone size, we stratified pts into favourable, neutral and adverse groups of the current and new model respectively (Tab.1) . A statistically significant difference in OS (p<0.05) among the 3 groups was observed only adopting the novel model. In addition, our in depth cytogenetics analysis showed ITH in 20 pts (35%), revealing the four recurrent CLL abs both as clonal and subclonal. Two mechanisms of clonal evolution were highlighted: linear, when a single clone sequentially acquires abs during tumor expansion, and branching, when two clones coexist and evolve in parallel.

Conclusions: We show that: a) the new stratification algorithm is superior to conventional model in predicting survival in patients with CLL; b) the concurrent cytogenetic abs and clone size should be comprehensively considered to determine the prognosis for CLL pts; c) searching ITH at diagnosis and/or at time of first treatment could predict clonal evolution and the generation of therapy-resistant subclones. In conclusion, we confirmed the crucial role of cytogenetic analysis for the routine clinical management of CLL and the importance of longitudinal cytogenetic study.

Table 1: Summary of FISH results in 58 CLL patients. **Favourable groups**: patients without any cytogenetic aberration or with only del(13q); **Neutral group:** patients with +12; **Unfavourable group**: patient with del(11q) and/or del(17p); **NEW Favourable group**: patients without any aberration or with only del(13q) or with a minor unfavourable clone (difference rate between major and minor clone of 30%); **NEW Neutral group:** patients with +12 and/or del(13q) with fewer than three aberrations; **NEW Unfavourable group**: patients with one poor clone, a major poor clone, or simultaneous 3 abnormalities. In addition, **in bold** patients with Intratumoral genetic heterogeneity.
Abbrevations: 13q-x1, deletion of one copy of 13q; 13q-x2, deletion of two copies of 13q; +12, trisomy 12; 17p-, deletion 17p; 11q-, deletion 11q; F: Favorable; N, Neutral; UN: Unfavorable.

Patients	% FISH abnormality	Stratification algorithm		Follow-up months
		Current	Novel	
CLL1	+12 [46%]; 11q- [10%]	UN	F	36
CLL2	13q-x1 [40%]; 13q-x2 [45%]; 17p- [10,5%]	UN	UN	35
CLL3	No abnormality	F	F	33
CLL4	+12 [90%]; 17p- [87%]	UN	UN	28
CLL5	No abnormality	F	F	27
CLL6	13q-x1 [12%]	F	F	27
CLL7	17p- [100%]	UN	UN	10
CLL8	13q-x1 [10%]; 13q-x2 [85%]	F	N	25
CLL9	+12 [80%]	N	N	46
CLL10	+12[75%]	N	N	24
CLL11	11q-[35%]; 17p-[20%]	UN	UN	23
CLL12	+12[85%]	N	N	22
CLL13	13q-x1 [75%]; 17p-[12%]	UN	F	22
CLL14	13q-x1 [70%]	F	F	22
CLL15	+12 [68%],17p-[14%]	UN	F	22
CLL16	+12[67%]	N	N	22
CLL17	13q-x1 [55%]; 13q-x2 [28%]; 17p [14%]	UN	UN	19
CLL18	13qx1 [93%]	F	F	19
CLL19	+12 [47%]	N	N	18
CLL20	13q-x1[20%]; 17p-[11%]	UN	UN	18
CLL21	+12 [74%]	N	N	7
CLL22	No abnormality	F	F	18
CLL23	13q-x1 [94%]	F	F	16
CLL24	13q-x1 [79%]	F	F	15
CLL25	13q-x1 [29%]	F	F	15
CLL26	13q-x1 [58%]; 13q-x2 [48%]	F	N	14
CLL27	13q-x1 [19%]; 11q- [87%]	UN	UN	14
CLL28	13q-x1[78%]; 13qx1 [11%]; +12[11%]; 17p- [43%]	UN	UN	10
CLL29	13q-x1 [79%]; 11q- [20%]	UN	F	12
CLL30	13q-x1 [51%]	F	F	11
CLL31	13qx1 [95%]; 17p- [12%]	UN	F	11
CLL32	No abnormality	F	F	9
CLL33	+12 [65%]	N	N	9
CLL34	13q-x1 [66%]; 11q- [61%]	UN	UN	8
CLL35	13q-x1 [90%]	F	F	6
CLL36	+12 [65%]	N	N	7
CLL37	13q-x1 [95%]	F	F	7
CLL38	13q-x1 [10%]; 11q- [90%]; 17p- [10%]	UN	UN	4
CLL39	13q-x1 [52%]; +12 [72%]; 11q- [18%]; 17p- [20%]	UN	UN	1
CLL40	13q-x1 [11,5%]; +12 [40%]; 11q- [53%]; 17p- [10,5%]	UN	UN	3
CLL41	13q-x1 [72%]	F	F	3
CLL42	13q-x1 [14%]; +12 [12%]; 11q- [48%]	UN	UN	2
CLL43	13q-x1 [70%]	F	F	10
CLL44	13qx1 [52%]; 11q- [15%], 17p- [82%]	UN	UN	2
CLL45	13q-x1 [65%]	F	F	48
CLL46	No abnormality	F	F	47
CLL47	+12 [65%]	N	N	47
CLL48	No abnormality	F	F	47
CLL49	13q-x1 [16%]	F	F	46
CLL50	13qx1 [30%]; 13q-x2 [70%]	F	N	47
CLL51	13q-x1 [64%]; 17p-[12%]	UN	F	46
CLL52	No abnormality	F	F	44
CLL53	17p- [58%]	UN	UN	44
CLL54	13qx1 [79%]	F	F	43
CLL55	13q-x1 [80%]	F	F	42
CLL56	13qx1 [60%]; 11q- [51%]; 17p- [10%]	UN	UN	39
CLL57	13q-x1 [32%]; 13q-x2 [10%]; 17p- [20%]	UN	UN	38
CLL58	No abnormality	F	F	37

PO089

CD81 NEGATIVE EXPRESSION PREDICTS A POOR CYTOGENETIC AND PROGNOSTIC RISK GROUP IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction: Several factors can predict the outcome in chronic lymphocytic leukemia (CLL), including immunophenotipic features. The expression of CD49d and CD38 represented the most important prognostic markers in CLL but no further markers have been investigated among those newly used. On the other hand, no markers seem to predict cytogenetic aberrations. CD81 is a tetraspasmin widely espressed on B cells and weakly expressed on CLL cells. Immunophenotipic studies of CD81 expression in patients with CLL are scanty and its value in predicting cytogenetics lesions as well as the outcome remains unknown. Thus, the aim of this study was to evaluate the potential role of CD81 in indentifying different cytogenetic and prognostic risk groups in CLL.

Methods: Samples of bone marrow from 71 patients with CLL were investigated for the surface expression of CD81 reported as percentage of CLL cells expressing the antigen and MFI. Thirty-one patients were treated and studied for minimal residual disease (MRD).

Results: The best cut-off points for CD81 were sought by constructing ROC curves, so that values greater than 20% of CLL cells and 530 by MFI were considered positive samples (CD81 pos) while the expression below these levels defined negative samples for this marker (CD81 neg). CD81 neg samples were significantly associated with unfavorable cytogenetic aberrations (67% vs 33%) such as 17p and 11q deletions, while CD81 pos samples were associated with favorable cytogenetic aberrations (96% vs 4%) such as 13q deletion and +12 trisomy (p=0.000). The majority of patients showing a negative cytogenetic profile with respect to previous aberrations had CD81 pos CLL cells (72% vs 28%)(p=0.000). There was an agreement between CD81 neg and CD38 positive expression (p=0.002). No significant correlations were found between CD81 expression and clinical features of CLL. When the only patients who underwent chemotherapy were considered, a significant association was showed between CD81 neg patients and a positive MRD (p=0.015). Interestingly, CD81 neg patients showed a significantly lower disease free survival (DFS) than those CD81 pos (p= 0.038).No significant associations were found between CD81 expression and the overall survival.

Conclusions: The absence of CD81 expression on CLL cells identifies patients who had unfavorable cytogenetic aberrations and a lower DFS compared to patients with positive CD81 expression, thus confirming the predictive role of CD81 in CLL

PO090

IMMUNOLOGICAL RECONSTITUTION AND PLATELET DYSFUNCTION DURING IBRUTINIB TREATMENT IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA. CLINICAL AND LABORATORY CHARACTERIZATION: MONOCENTRIC EXPERIENCE

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Introduction: Chronic lymphocytic leukemia (CLL) is characterized by an high rate of infectious complications probably secondary to immune dysfunction. Ibrutinib (IBR) in CLL seems to generate partial reconstitution of normal B cells and humoral immunity, expecially increase in IgA. IBR is associated with bleeding events, usually mild (grade1-2), rarely severe (grade 3-4). A defect of platelet function, namely an inhibition of Btk-and Tec-mediated signaling downstream platelet glycoproteins GPVI and GPIb, probably cause bleedings.

Methods: We report our experience on immunological reconstitution and platelet dysfunction observed in 27 pts treated with IBR for progressive naïve CLL with del17p/TP53 or with relapsed or refractory disease. All pts before and 1, 3, 6, 9 and 12 months after beginning IBR was studied for the immunological reconstitution by measuring IgG, IgM, IgA, CD3 T lymphocytes (lymph), CD4 and CD8 T-helper and T-suppressor subset, B lymph CD19+ and NK lymph CD16/56+ by flow cytometry and for the platelet dysfunction by light transmission aggregometry using platelet-rich plasma and ADP, PAR1-AP, Collagen, Arachidonic Acid, ristocetin as platelet agonists. No pts received concomitant antiplatelet or anticoagulant.

Results: During the study period the immunoglobulin levels did not show any change and the median values were constantly belove the normal range. Immunological reconstitution showed a rapid increse of CD19+ lymph above the normal range after 1 month of IBR and rapidly decreased to normal values. CD3+ lymph remained into the normal from baseline to 12 months during IBR, the subset CD4+ decreased from months 3 to 12 with median values belove the normal range, on the contrary CD8 showed a progressive increase in the same period with median values into the normal range. CD16/56+ were into the normal range during the study period. In 18 pts we observed only grade I or II bleedings (bruising, petechiae, intraocular hemorrhage, rectal bleeding); no pts needed IBR interruption or dose reduction. All pts during IBR showed severe impairment of collagen induced aggregation. On the contrary, the aggregation by low-dose ADP significantly improved; while the aggregation by other agonists was unchanged. In 18 pts the vWF:Ag and RiCo were high at the onset of the disease and reduced up to normal values under IBR.

Conclusions: In our pts there was no improvement of humoral immunity, even after 12 months of IBR. As expected, B-lymphocytes rapidly decreased up to the normal range after 1 month; T-cell compartment remained within normal values, with a trend toward an increase of T-Suppressor and a reduction of T-Helper after 12 months of IBR, which contributes to immune reconstitution. We observed only minor bleedings. IBR caused a severe impairment of collagen-induced aggregation, while ameliorate of ADP-induced aggregation. Finally, pts under anticoagulant or antiplatelet might need be carefully monitored by clinical and laboratory evaluation.

Monoclonal Gammopathies and Multiple Myeloma 2

PO091

ANTI-PROLIFERATIVE AND CYTOTOXIC EFFECTS OF AZORELLA GLABRA CHLOROFORM FRACTION ON MULTIPLE MYELOMA CELLS

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Introduction: Plant extracts play an important role in antitumor drug discovery because they show large structural diversity, low toxicity, favourable profile of absorption and metabolism and can interfere with proliferation, differentiation and apoptosis processes of malignant cells. In search of novel plant derived anticancer agents, it has been reported that methanolic extract of Azorella compacta induces apoptosis of human leukemia HL60 cells. Given these preliminary results, we tested the cytotoxic effect of 6 different A. glabra (AG) extracts and of different subfractions obtained from the most active fraction on acute myeloid leukemia (AML), natural killer cell leukemia (NKL) and multiple myeloma (MM) cell lines.

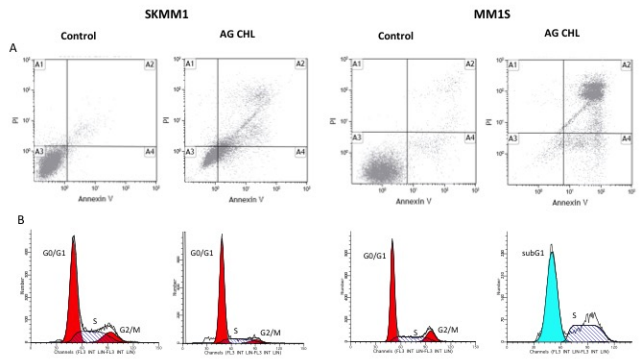

Figure 1: AG CHL extract treatment induced apoptosis and cell cycle arrest in G0/G1 phase in SKMM1 and MM1S cell lines. (A) Flow cytometric analysis of AG CHL extract-induced apoptosis in SKMM1 and MM1S cells using AnnexinV/Propidium Iodide (PI) double staining. (B) Cell cycle analysis performed labeling SKMM1 and MM1S cells with PI/RNase staining solution. Data were recorded 48h after treatment with 50µg/ml of AG CHL extract. AG: *Azorella glabra;* CHL: chloroform fraction.

Methods: Dried whole plant of AG was extracted in methanol or 96% ethanol. Subsequently, crude ethanolic extracts were partitioned using solvents with increasing polarity: n-hexane, chloroform, ethyl acetate, butanol and water. KASUMI-1 (AML), KHYG1 (NKL), MM1S, RPMI8226 and SKMM1 (MM) cell lines were treated with 10, 50, 100 and 150µg/ml of 6 AG extracts for 24, 48 and 72h. Chloroform extract was separated by silica gel column chromatography, fractions with similar chromatographic pattern were combined and viability test on RPMI8226 was performed. Cell viability was assessed by MTS assay and was calculated as percentage of viable cells compared to control (DMSO). EC50 values were obtained by GraphPad Prism software. Apoptosis was evaluated by analysis of AnnexinV/Propidium Iodide (PI) stained cells and cell cycle analysis performed labeling with PI/RNase staining solution. Stained samples were acquired by NAVIOS cytometer. Peripheral blood mononuclear cells from healthy donors (HD-PBMCs) were isolated by Ficoll gradient separation and used as controls.

Results: Viability test showed that chloroform fraction had major effect in term of reduction of cell viability and its effect was dose and time dependent. MM cells were the most sensitive to chloroform treatment (EC50 was of 40,8 µg/ml on SKMM1, 63,1µg/ml on MM1S and 20 µg/ml on RPMI8226). Importantly, the effect on HD-PBMC viability was negligible. Furthermore, chloroform fraction induced apoptosis and cell cycle arrest in G0/G1 phase in MM treated cells (Figure 1). Further purification of the chloroform fraction was performed and among the 12 sub-fractions obtained, one had a greater cytotoxic effect on RMPI8226 cells respect to chloroform fraction emphasizing an enrichment of bioactive compounds.

Conclusions: This preliminary study demonstrates that chloroform AG extract is able to reduce cell viability and to induce apoptosis and cell cycle arrest in G0/G1 phase in MM cell lines. Given the negligible effects on HD-PBMC this extract and its sub-fraction warrants further investigation in order to candidate AG as source of new therapeutic drugs in the landscape of novel targets for MM treatment.

PO092

MESANGIOGENIC PROGENITOR CELLS ROLE IN MULTIPLE MYELOMA MICROENVIRONMENT

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Introduction: Mesangiogenic progenitor cells (MPCs) are a cell population isolated from human bone marrow (hBM), showing the ability to differentiate into mesenchymal stromal cells (MSCs) and retain an angiogenic potential. The MPC mesengenic differentiation includes two consecutive steps: a first differentiation into "early MSCs" (also called P1-MSCs) with the activation of Wnt-5/calmodulin pathway, then a terminal differentiation into "late MSCs" (also called P2-MSCs) independent from this pathway. Indeed, only the early phase could be blocked by Calmidazolium Chloride (CLMDZ), a potent calmodulin inhibitor. Similarly, the MPC angiogenic differentiation is not affected by CLMDZ, but is inhibited by Bortezomib. For their peculiar features, MPCs can be thought to be involved in the pathogenesis and progression of multiple myeloma (MM). Previous *in vitro* experiments, performed on BM samples from newly diagnosed MM patients, showed that both mesengenic and angiogenic differentiations were impaired by Bortezomib while CLMDZ did not affect any differentiation. This data suggests that possibly MPCs would be restricted to an angiogenic fate losing the mesengenic potential in the pathological setting. In this study we evaluate the angiogenic sprouting of MPC-derived P1-MSCs, these cells, normally, do not retain any angiogenic potential, as previously demonstrated.

Methods: BM samples were obtained from 15 newly diagnosed MM patients after written consent. We isolated MPCs, as previously described, from each sample applying a medium for MSC expansion to perform mesengenic differentiation. After 6 days of culture, P1-MSCs were detached and two 3D-spheroids were produced by the hanging drop method. The spheroids were then plated on Matrigel thick gel and cultured in EGM-2 endothelial growth medium for 7 days. Sprouting distance was then measured and the mean values, obtained from three different observers, were recorded and analyzed by t-test.

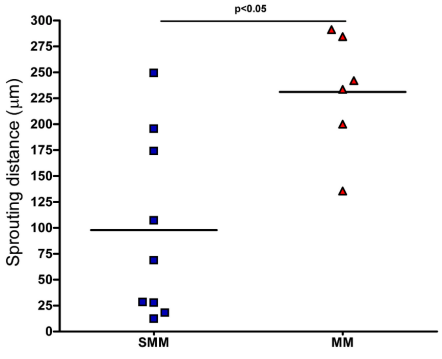

Figure 1.

Results: Nine of 15 patients have a mean sprouting value of 97.89 ±29.49 μm while the other 6 have a mean value of 231.12 ±23.57μm, the statistical analysis demonstrates a significative difference ($p<0.05$) between the groups (Figure 1). Surprisingly, higher sprouting values have been detected in all patients with an active stage of disease, 5 of 6 patients also reported osteolytic lesions. Conversely a reduced sprouting distance has been measured in patients affected by smoldering MM (SMM).

Conclusions: Multiple Myeloma as a BM niche disease, affects the microenvironment enhancing angiogenesis and reducing bone formation. The progression from a non-active stage of disease to a symptomatic phase is characterized by the "angiogenic switch". Our results suggest that disease in symptomatic stage probably forces MPCs toward the angiogenesis, this restriction is probably accompanied with a reduced mesengenesis *in vivo*. MPCs seem to be involved in the "angiogenic switch" and could represent a new therapeutical target.

PO093

CHANGE OF WILLEBRAND FACTOR AND VWF:RCO VALUES AFTER CARFILZOMIB INFUSION

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Introduction: Carfilzomib, a 2nd generation proteasome inhibitor, is today one of the most widely used drugs in multiple myeloma therapy.

The most common side effects concern the cardiovascular system with hypertension crisis that may occur immediately after its administration; in this regard an increase of BNP has been reported after Carfilzomib infusion.

Aim of the study: In this study, we want to evaluate whether Carfilzomib infusion is associated with an increase in the plasma level of the Willebrand factor and VWF:RCo activity.

Materials and methods: We followed 7 patients with multiple myeloma on 2nd or 3rd line therapy with Carfilzomib (KD or KRD) and we evaluated the value of Willebrand Factor and VWF:RCo at time 0 and after 90 minutes from each administration. We have chosen this time point on bases of Desmopressin test used in the diagnosis of type 1 Von Willebrand disease, in which the desmopressin infusion significantly increase the Willebrand factor after 60-90 minutes. We performed a total of 35 tests with a minimum of 2 and a maximum of 11 tests per patient.

Results: In 6 patients the baseline value of Willebrand factor and VWF:RCo were always higher than normal values (mean of 238% for Willebrand factor and 193% for VWF:Rco), only in 1 patient the baseline values were in normal range. The 90-minute evaluation showed a wide variability of results with respect to baseline, intra and inter-patients, with high concordance between Willebrand factor and VWF:Rco in all evaluations. In 14/35 tests the value of Willebrand factor and VWF:Rco increased after the administration of the drug; the maximum variation with respect to T0 was 84% for Willebrand factor and 69% for VWF:Rco The patient treated with the KD protocol showed the highest number of tests with increase in T90 values (55,4%); the average value of Willebrand factor went from 213 to 226 and VWF:Rco from 193 to 216. In the same patient we found the more pronounced variation of arterial pressure.

Conclusions: Our study shows that Carfilzomib infusion can be followed by a change in plasma levels and Willebrand factor level and functionality. It remains to investigate whether this variation has clinical significance and whether it has correlations with the side effects on the cardiovascular system. If this correlation existed, it would be necessary to evaluate whether the Willebrand variation has a pathogenetic significance or if it is only a consequence of the vascular stress induced by the drug.

PO094

PRECLINICAL ACTIVITY OF NOVEL BRUTON'S TYROSINE KINASE INHIBITOR ARQ531 ON MULTIPLE MYELOMA CELLS

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Background: Multiple myeloma (MM) is an incurable plasma cells malignancy, which typically responds to current treatment, eventually relapses and leads to patients' death finally. Therefore, identification of novel therapeutic markers and development of innovative treatment strategy remains an unmet medical need. Recent data suggest activation of Bruton tyrosine kinase (Btk) as essential for plasma tumor formation with its elevated levels associated with poor prognosis. As a result, several Btk-inhibitors have been investigated but, differently from other lymphoproliferative disorders, clinical efficacy of these drugs on MM still lacks. Here we report preclinical data of ARQ 531, a novel small molecule Btk inhibitor, on MM cells.

Material and Methods: We utilized a panel of human MM cell lines both sensitive (MM.1S, RPMI-8226, NCI-H929, LP1, KMS34, KMS20, KMS11, KMS12-BM, KMS12-PE, KMS27, MR20, OPM2, MOLP8, KMS26, U266) as well as resistant to anti-MM drugs (DOXO40 and IMiDs-resistant NCI-H929) and purified tumor cells obtained from NDMM and RRMM patients. Cell viability and apoptosis were investigated using MTT-based assay and Annexin V/PI staining, respectively. Next, the cytotoxic effect of such treatment was also investigated on MM patients-derived BM stromal cells (BMSC) co-coltured with Nanoluciferase-expressing MM cell lines (NCI-H929). Immunoblot analyses were performed using specific antibodies. Finally, combination-treatments with different anti-MM drugs were also investigated by employing an isobologram analysis (CalcuSyn software program).

Results: ARQ531 treatment inhibited cell viability of MM cell lines (n=16) with IC50 value of 14±1 μM compared with 39±0.6 μM of Ibrutinib (standard control). Consistent with these data, ARQ531 increased also the apoptotic rate of MM-tested cells than ibrutinib. Importantly, anti-MM effects of this molecule were not abolished in presence of bone marrow stromal cells (BMSCs), and, more significantly it showed greater therapeutic window than standard. Next, combination strategies were also tested with IMiDs-cotreatment (pomalidomide and lenalidomide) resulting in superior synergistic effect ($P<0.01$, $CI < 1$) than those observed with other combinations (bortezomib, carfilzomib, melphalan and bendamustine). Furthermore ARQ531 activity was not affected by drug resistance status as observed on Len/R cells, which showed similar IC50 value than isogenic control. Ongoing mechanistic studies will further provide insights into biological events leading our observations.

Discussion: The novel BTK inhibitor ARQ 531 is a highly potent Kinase inhibitor with promising activity against MM as observed in preclinical models. Overall, our data support the feasibility and therapeutic relevance of ARQ531 on this malignancy, regardless of specific genomic landscape, providing also the rationale for its evaluation in MM clinical protocols.

PO096

ERWINIA CHRYSANTEMI-DERIVED L- ASPARAGINASE STRONGLY ENHANCES ANTI-MM DRUGS ACTIVITY BY DEREGULATING METABOLIC CELL PROGRAM

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Introduction: Human tumors are highly reliant on Glutamine (Gln) as it sustains metabolic processes and macromolecules synthesis. Such dependence defines a "Gln-addiction" status which has been described also for multiple myeloma (MM) cells which are extremely sensitive to Gln depletion due to a deficiency of Gln synthetase. L-Asparaginase, a key drug in ALL treatment, is an enzyme that catalyzes the hydrolysis of asparagine (Asn) and Gln. Here we explored MM cells sensitivity to pharmacological Gln depletion achieved by Erwinia chrysantemi-derived L-ASP treatment as single agent and in combination with different anti-MM drugs. We chose E. chrysantemi L-ASP due to its high glutaminase activity.

Materials and Methods: The IC50 value of L-ASP was determined by MTS assay in a panel of human MM cell lines (HMCLs) harboring different genetic background as well as on primary CD138^pos cells derived from NDMM patients. Cell death analysis was measured with Annexin V/Propidium Iodide (AV/PI) staining followed by flow-cytometric analysis. L-ASP treatment effect on cell viability and mitochondrial membrane potential was evaluated by FACS analysis following PI and TMRE staining, respectively. Combination drugs screening with L-ASP plus different anti-MM drugs, such as the proteasome inhibitor carfilzomib (KAR) and the alkylating agent melphalan (MEL), was also investigated with an isobologram analysis (CalcuSyn software program). Finally, western blot analysis was used to fully elucidate biological features of our findings.

Results: L-ASP treatment showed potent anti-tumor activity in all HMCLs tested with an IC50 value ranging from 0,03 U/mL to 0.2 U/mL. Importantly, such effect was also observed in primary MM cells (IC50 =0.06 U/mL). Cell cycle analysis revealed an early (24 h) but irreversible "metabolic shutdown" in MM cells treated with L-ASP whilst the onset of mitochondrial stress was evident later (48-72h). To corroborate Gln role, MM cells viability in presence of different culture medium conditions was measured. Remarkably, Asn depletion alone did not modify significantly MM cells viability, instead it strongly contributed to L-ASP induced toxicity of Gln-starved cells. Co-treatment experiments showed massive increase of L-ASP anti-MM activity in presence of low doses of KAR and MEL, with Combination Index value less than 1 in almost all tested drugs-concentrations. Notably, IL6 or IGF-1 addition did not reduce anti-MM activity of combination suggesting that MM microenvironment does not affect efficacy of such approach. Finally, proteomic analyses revealed c-MYC down regulation as well as caspase-3 and PARP cleavage in co-treated cells pointing the crucial role of apoptosis in the observed synergism.

Conclusions: Our preliminary data show that E. chrysantemi L-ASP exerts a potent anti-MM activity, mainly due to its glutaminase activity, which results in a massive metabolic program deregulation. Addition of low-doses of commonly used anti-MM agents further increases L-ASP activity.

PO097

CD38 HANDLING AS STRATEGY TO MAKE MULTIPLE MYELOMA CELLS MORE SENSITIVE TO NAD+ DEPLETING AGENTS

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Background: Upregulation of NAD+ biosynthesis is needed to face the increased proliferation and metabolic processes of neoplastic cells and represents an important feature distinguishing these cells from their normal counterparts. As a result, the NAD+ biosynthetic apparatus arises as highly promising therapeutic target for tumors, as suggested by the use of nicotinamide phosphoribosyltransferase (NAMPT) inhibitors in a number of malignancies, including Multiple Myeloma (MM) and leukemia (Cea et al. Blood 2012; Cagnetta *et al.* Blood 2013; Cagnetta et al. CCR 2015; Soncini et al. Haematologica 2017). An emerging, pleiotropic therapeutic target in MM is the NAD+-degrading enzyme CD38, a surface protein highly expressed by tumor cells compared with other normal hematopoietic cells. As a result, several anti-CD38 monoclonal antibodies, including daratumumab and isatuximab, have been tested in clinical trials with promising Results: Overall, the available preclinical and clinical data clearly indicate a strong potential for CD38 as target in MM, with the expectation that, by simultaneously interfering with several aspects of carcinogenesis, CD38-targeted agents will lead to impressive clinical Results: Nevertheless, further steps need to be taken to allow a full exploitation of CD38 as a target in MM including a more thorough definition of its role in tumorigenesis and its metabolic dependencies that could be selectively targeted for therapeutic gain. Based on these assumptions, here we explored CD38 activity as innovative strategy to enhance the anti-tumor activity of NAMPT inhibition in Multiple Myeloma cells.

Methods: A panel of different MM cell lines and primary cells, both sensitive and resistant to conventional and novel anti-MM therapies, was used in the study. The effects of NAMPT inhibition was evaluated in presence of CD38-gene editing (loss/gain of approaches) by using CTG assay and Annexin-V/propidium iodide staining. Next, the anti-MM effects of chemicals affecting CD38 activity were also evaluated in combination with low doses of NAMPT inhibitors. Mechanistic studies were performed with western-blotting, enzymatic assays and gene expression profiling analysis.

Results and Conclusions: By using different approaches, we found that CD38 deregulation makes MM cells more vulnerable to NAD+ depleting agents. Ongoing mechanistic studies suggest the central role played by energetic metabolism in the observed synergism. Overall our data provide the mechanistic preclinical rationale to enhance anti-MM activity of NAMPT targeting agents, in order to both overcome drug resistance and improve patients outcome.

PO098

PREVENTING AND MANAGING THROMBO-EMBOLIC EVENTS (TE) IN MULTIPLE MYELOMA PATIENTS TREATED WITH THIRD GENERATION NOVEL AGENTS IN REAL LIFE

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In comparison with normal health population risk of venous thromboembolic event (VTE) in multiple myeloma (MM) patients is higher. A recent consensus on prevention and management of MM patients has shown that, among adverse events, thromboembolic complications are quite frequent during treatment with novel agents in clinical trials. It has been widely described that use of IMiDs such as thalidomide or lenalidomide but also alkylating agents or erythropoietin increase the risk of VTE, but little is known on next generation anti- myeloma drugs, such as pomalidomide, carfilzomib, daratumumab and elotuzumab in real life.

Thus, we aimed to evaluate the incidence of VTE in a cohort of 109 relapsed and/ or refractory MM patients treated with third generation novel agents in real life. From April 2013 up to now 109 patients were treated with novel agents: 69 patients were treated with pomalidomide and desamethasone (PomaD), 37 with carfilzomib, lenalidomide and desamethasone (KRd), 13 with daratumumab (Dara) and 6 with elotuzumab, lenalidomide and desamethasone (EloRd). 8 patients received pomalidomide and KRd, 6 received Pomalidomide, KRd and Dara, 1 received Pomalidomide and Dara, one patient were exposed to pomalidomide, KRd, daratand EloRd regimens, and 2 patients received KRd and Dara. 63 patiens were males (57%), median age was 69 (range 47-87), 58 were refractory (53%). Median of previous line of treatment was 3, range 1-11. Although standard prophylaxis, 4 patients treated with pomalidomide had a VTE consisting in VTP (2) and EP (2); they were treated with CardioAsa and when VTE was diagnosed with LMWH. Analysis of risk factors shows that among Poma Treated patients 42 had >1 patients-related risk factors, 31 >2 patients-related risk factors and

66>1 therapy-related risk factors. Among poma treated patiens with VTE all had >1 patients-related risk factors and 3 >2 patients-related risk factors and 4>1 therapy-related risk factors. In patients treated with KRd only 2 patients had a VTE (2 VTP) and XX had risk factors; they were treated with XX. Only dara treatment did not include standard prophylaxis.

Conclusion: real life observation of VTE revealed a low incidence in patients treated with novel agents. This is probably due to standard prophylaxis in case of therapies including immunomodulators or in case of patients with high risk for thromboembolic complications.

PO099

MINIMAL RESIDUAL DISEASE AND LOG-REDUCTION OF PLASMA CELLS EXPLAIN THE SUPERIOR VALUE OF DOUBLE AUTOLOGOUS STEM CELL TRANSPLANT IN YOUNGER PATIENTS WITH MULTIPLE MYELOMA

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Introduction: Although advances in the treatment of multiple myeloma (MM) have increased the frequency of complete response (CR), many MM patients still experience relapse, suggesting that MM hides a persistent disease, mostly undetectable to current diagnostic methodologies, and that new therapeutic strategies after single transplant are needed. The identification of minimal residual disease (MRD) after autologous stem cell transplant (ASCT) identifies high risk MM patients better than CR. Moreover, the role of double ASCT as new adjunctive therapeutic strategy remains to be elucidated. Here, we investigated the role of MRD and Log-reduction of plasma cells (PCs) in quantifying the degree of tumor reduction after any ASCT and in predicting the outcome.Methods: Bone marrow samples from 30 patients who underwent double ASCT were assessed by FC at different time points: post induction (MRD1 and LOG1), post first- (MRD2 and LOG2) and post- second (MRD3 and LOG3) ASCT. MRD (>0.01%) was evaluated by a six-color FC. Log – reduction was calculated as a logaritmic ratio between the PCs at presentation and PCs at each time of assessment. Results: A significant difference was evidenced among the three time points from ANOVA test for both LOG-reduction ($p<0.001$) and MRD ($p=0.005$). In particular, LOG3 was significantly greater than LOG2 ($p<0.001$) and LOG1($p<0.001$). Similarly, MRD achieved after double ASCT was deeper than MRD achieved after single ASCT ($p=0.005$) and after induction($p<0.001$). Then, frequency of MRD positive patients after double ASCT was significantly lower than that found after the first ASCT (n= 15 vs n= 23,$p=0.008$) and after induction (n=15 vs n=27, $p=0.004$). When the survival analysis was considered, a significant reduction of PFS was observed in patients belonging to an unfavorable cytogenetics risk group ($p<0.001$) and patients showing a MRD over 0.01% ($p=0.001$) as well as a Log-reduction lower than 2.57 ($p=0.018$) after double ASCT . Presence of MRD after single transplant also predicted a shorter PFS ($p=0.018$), defining also this time point as prognostic for MRD assessment Conclusions: Our results by FC sustained the double ASCT as the goal treatment strategy in MM because of a deeper reduction of PCs, a higher frequency of MRD positive patients and a longer PFS compared to single ASCT.

PO100

POMALIDOMIDE-DEXAMETHASONE IN THE MANAGEMENT OF HEAVILY PRETREATED MULTIPLE MYELOMA

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Pomalidomide is a new generation IMID, with a very good compliance, thanks to oral administration, which can be used also in heavily pretreated patients, in a domestic setting. In this retrospective observational trial, It has been evaluated efficacy and tolerance of pomalidomide plus dexamethasone (PD) as salvage regimen in heavily pretreated patients with relapsed and refractory MM (rrMM), whose prognosis is particularly severe. 29 patients (17 M/12 F), with rrMM, median age at diagnosis 69 years (r. 52-84), and median age at start of treatment 76 years (r.56-89) treated with several lines of treatments (median 7, r. 2-11), every refractory to all the drugs previously received (also Bortezomib, Thalidomide and Lenalidomide), received Pomalidomide-Dexamethasone (Pomalidomide 4 mg for 21 days, Dexamethasone 40 mg days 1,8,15,22, pegfilgrastim day +8) every 28 days, until progression ISS was equally distributed, and cytogenetic was evaluable in 14 patients, and in particular three del13q and one t(11;14) were present. All the patients had previously been treated with schedule containing bortezomib and IMIDs. 55% (16/29) of them had undergone at least to a single ASCT. All patients were relapsed and refractory to last therapies received before PD. Pomalidomide was well tolerated, with grade 3 anemia in 48% (14/29) of patients, 31% (9/29) grade 3 neutropenia (pegfilgrastim in primary prophylaxis was given, no hospitalization was required, no septic shocks were observed), 27% (8/29) grade 3-4 thrombocytopenia without hemorrhagic events and transfusion-dependence. No severe extra-hematologic toxicity was observed. According to IMWG, ORR1 (≥PR) was 44% (13/29: 3 CR, 4 VGPR, 6 PR), but, considering that we are evaluating a cohort of heavily pretreated patients without any other alternative treatment, with really poor prognosis, another parameter should be considered, ORR2 (≥SD), considering stable disease as a successful result in progressive MM. ORR2 was 76% (22/29: 3 CR, 4 VGPR, 6 PR, 8 SD). These can be considered as impressive result in this subset of patients. Oral treatment gives a really good compliance, in frail and unfit patients, and response, when present, is always really fast (median time to response: 2 months (r.1-6)), median OS from diagnosis was 92 months (range 21-228), median OS from start of pomalidomide was 8 months (range 1-18). Pomalidomide-dexamethasone has shown significant efficacy and a very good compliance, thanks to oral administration, in a particularly severe setting of heavily pretreated patients, relapsed and refractory to all available therapeutic resources.

PO101

BORTEZOMIB, LENALIDOMIDE AND DEXAMETHASONE IN THE MANAGEMENT OF RELAPSED AND REFRACTORY MULTIPLE MYELOMA

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Bortezomib, Lenalidomide and Dexamethasone is one of the best option for frontline treatment, approved in USA but not available in Italy However, it can show interesting results also in relapsed and refractory patients, thanks to the synergistic effect of these agents. In this retrospective observational study, it has been evaluated the safety and efficacy of the combination of bortezomib plus lenalidomide plus dexamethasone (VRD) in patients with relapsed and refractory Multiple Myeloma (rrMM). 29 patients (19 M, 10 F), with rrMM, median age 64 years (range 38-79), were treated with the VRD regimen (Bortezomib 1.3 mg/sqm days 1,4,8,11; dexamethasone 20 mg days 1, 2, 4, 5, 8, 9, 11, 12 and oral lenalidomide 25 mg daily on days 1-21), with a median of 6 cycles (range 1-21). Patients had previously received 3 median (range 1-6) lines of therapy. 83% (24/29) of them had undergone to autologous

SCT.

According to IMWG, ORR was 79.3% (23/29: 6 CR, 5 VGPR, 7 PR, 5 SD). Median time to response was 3 months (range 1-6), median OS from diagnosis was 56 months (range 12-221).

Bortezomib-lenalidomide-dexamethasone was well tolerated, with grade 1-2 anemia in 5 patients, successfully managed with ESAs, and, thanks to the way of administration, also compliance is good. Peripheral neuropathy was seen in 48% (14/29) patients.

Bortezomib-lenalidomide-dexamethasone triplet, thanks to a notable proved synergistic mechanism of action between bortezomib and lenalidomide, had shown significant efficacy in severe setting of heavily pretreated patients, relapsed and refractory to bortezomib and lenalidomide.

Lymphomas

PO102

CEREBROSPINAL FLUID FLOW CYTOMETRY FOR DIAGNOSIS AND MONITORING OF NHL: A REGINA ELENA NATIONAL CANCER INSTITUTE EXPERIENCE

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Introduction: Central nervous system (CNS) involvement in non-Hodgkin's lymphoma (NHL), significantly more frequent in aggressive subtype, has an incidence of 5-9% and represents a negative prognostic factor. Compared to cerebrospinal fluid (CSF) cytology, the gold standard for leptomeningeal infiltration diagnosis, flow cytometry (FC) has shown to be a more sensitive diagnostic tool for CNS infiltration. We report the role of FC in diagnosis and monitoring of CSF infiltration in a large cohort of NHL patients.

Methods: From January 2007 to June 2012, a total of 179 CSF samples, corresponding to 131 NHL patients, were characterized by FC and compared to cytology to evaluate the possible CNS involvement. Peripheral blood FC study was performed on 75 patients.

Results: According to the WHO classification, 83 were diffuse large B cell, 16 mantle cell, 8 peripheral T cell, 6 follicular, 5 Burkitt, 5 primary CNS, 4 anaplastic ALK+, 2 lymphoblastic, 1 NK and 1 dendritic cell NHL. A positive CSF cytology was documented in 3.8% of cases (5/131 patients), by contrast 6.9% of cases (9/131 patients) were positive by FC. None of the patients positive for cytology were negative by FC ($p < 0.0001$). In the 5 patients positive both by cytological and FC analysis, the percentage of pathological cells identified by FC was significantly higher compared to the 4 patients with negative cytology and positive FC (90% vs. 19.5%). A trend was observed suggesting that cytology is capable of CSF infiltration diagnosis only for high infiltration rates (Mann-Whitney U test $P=0.086$). Moreover, 48 samples were collected for minimum residual disease monitoring. In these patients, cytology was positive in 18.8% and FC positive 39.6% of cases (19/48 patients) ($P = 0.006$). Focusing on samples with low cell count (number of events lower than 50th and 25th percentile on immunophenotypic examination), cytological examination did not show positive cases (0/24; 0/12), by contrast FC identified 6 and 3 positive samples in the respective subgroups. The blood analysis showed the presence of circulating neoplastic cells in 8/75 patients with a correlation between blood and CSF involvement ($P = 0.014$). A T lymphocytes population was documented in all the CSF samples (100%), flanking the lymphoma cell in the positive cases.

Conclusions: Compared to cytology, FC confirmed to be a more sensitive approach for CSF infiltration in NHL both in patients at diagnosis and for minimal residual disease assessment with a relevant diagnostic advantage in CSF samples with low cell count. A positive correlation was found between the presence of peripheral blood circulating lymphoma cells and CSF infiltration. As previously reported by our group in breast cancer neoplastic meningitis, a side population of reactive T lymphocytes was observed in all the case, documenting an active mechanism of T-lymphocytes migration to CSF.

PO103

MIR-22 SIERICO UN NUOVO MARCATORE PROGNOSTICO NON INVASIVO NEI PAZIENTI AFFETTI DA LINFOMA DIFFUSO A GRANDI CELLULE B: RISULTATI PRELIMINARI DI UNO STUDIO PROSPETTICO

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Introduction: Bodily fluids are an important source of information in several diseases analyzable by liquid biopsies, representing minimally invasive methods for diagnostics and prognosis. Blood extracellular miRNAs are under investigation as novel biomarkers. While tissue miRNAs in DLBCL patients have been extensively studied, only few reportsevaluated the role of circulating/serum miRNA as potential prognostic factors. Our laboratory has previously performed a pilot study on a training cohort of 36 DLBCL patients where we observed that miR-22 serum levels at diagnosis were significantly correlated with progression free survival of DLBCL patients. Moreover an in silico pathway analysis has been performed showing that miR-22 target genes are involved in different important pathways such as p53 and PI3K/AKT signaling. Our current aimsare a) to validate theserum miR-22as novel and reliable prognostic biomarker in DLBCL; b) identify/dissect the molecular pathways of miR-22 involve the pathogenesis of the DLBCL //molecular role of miR-22 on the DLBCL pathogenesis through pathways analysis.

Methods: This is an on-going prospective study on newly diagnosed de novo DLBCL patients uniformly treated with 6 courses of R-CHOP. Serum samples of patients were collected at diagnosis and after the end of treatment. The expression profile of miR-22 was evaluated by qRT-PCR in serum samples collected at diagnosis and after treatment in a validation cohort of DLBCL patients (power calculations to determine the required sample size was estimated based on our previous data to achieves a 90% statistical power at 0.01 significance level). To investigate the molecular pathways of miR-22 involve the pathogenesis of the DLBCL, we are currently analysing the expression levels of miR-22 targets genes, identified by silico analysis, in DLBCL formalin fixed and paraffin embedded tumour tissue samples comparing them with miR-22 expression in matched samples.

Results: (a) Our ongoing experiments show that serum miR-22 expression is significantly correlated with patients clinical outcome and is higher at diagnosis in patients unresponsive to treatment when compared with responsive patients, thus confirming our previous Results: Regarding aim (b),our current preliminary data show that miR-22 expression level in tumour samples is inversely correlated with that of its target genes validating the results of in silico analysis.

Conclusions: Our preliminary results suggest that miR-22 is of potential interest as novel and reliable prognostic biomarker in DLBCL and the identification/dissection of its molecular pathways involved in DLBCL pathogenesis can pave the way to the development of targeted therapy for specific subgroups of DLBCL patients.

PO104

CK2A IS OVEREXPRESSED, ACTIVE AND TARGETABLE WITH SILMITASERTIB IN CLASSICAL HODGKIN LYMPHOMA

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Introduction: Most patients with HL are cured with first line therapy however, treatment of relapsed patients still represents as unmet medical need requiring the development of new effective drugs. CK2 is a pleiotropic kinase consisting of 2 catalytic(a) and 2 regulatory(b) subunits that sustains cancer signaling cascades through the activation of NF- B, PI3K and JAK/STAT pathways. Despite it has been demonstrated that CK2 confers resistance to apoptosis and promotes cell cycle progression in non-Hodgkin lymphomas (NHL), this protein has not yet been investigated in Hodgkin lymphoma (HL). Considering that NF-kB, JAK/STAT and PI3K pathways are key players in HL, it is likely that CK2 plays a pivotal role in the pathogenesis of this disease. The aim of this study was to dissect the expression and the function of CK2 in HL

Methods: Experiments were performed and replicated at least for five times employing 4 HL cell lines L-428, L-540, KM-H2 and HDML-1 cultured in RPMI for 24h, 48h and 72h with or without the CK2 inhibitor silmitasertib. Normal B-cells were obtained from age-matched healthy subjects. CK2 , CK2 , RelA-Serine (S)529, RelA, PARP, AKT-S473, AKT, STAT3-S727, STAT3 and tubulin expression levels were evaluated by western blot analysis (WB) and/or immuno-fluorescence (IF). Apoptosis was assessed by Annexin V/Propidium iodide (AV/PI) assay and PARP cleavage by WB. Immunohistochemistry (IHC) for CK2a and CK2b was performed on formalin-fixed/paraffin-embedded sections of lymph-nodes from patients with HL, as well as indolent and aggressive NHL. Data were considered statistically significant when p values were <0.05

Results: By WB and IF we found that all the 4 HL cell lines expressed higher levels of CK2a, but not of CK2b, as compared with normal B lymphocytes. These data were confirmed by IHC on primary lymph-nodes derived from patients with HL, showing that CK2a but not CK2b was highly expressed in Reed-Sternberg cells. No clinical data correlated with CK2a expression. This unbalance between a and b subunits was not observed in follicular lymphoma, DLBCL and Burkitt lymphoma cases ($p<0.01$). We also analyzed CK2 subcellular localization and found that the a subunit was present both in the nucleus and in the cytosol, while the b subunit was present only the cytosolic compartment. In order to evaluate the activity of CK2, we performed WB analysis of the phosphorylation status of CK2 substrates. AKT, RelA and STAT3 were constitutively phosphorylated in HL at their activatory S-residue (S473, S529, and S727 respectively). Treatment of HL cell lines with silmitasertib caused down-regulation of AKT-S473, time and dose-dependent apoptosis as assessed by AV/PI test and PARP cleavage ($p<0.01$)

Conclusions: We demonstrated that CK2a is overexpressed, active and induces key pro-survival signals in HL. Chemical inhibition with silmitasterib was able to induce apoptosis of HL cell lines. These preliminary data suggest that CK2 could play a pivotal role in HL

PO105

MOMELOTINIB AND CITARINOSTAT: CO-TARGETING JAK2 / STAT3 AND HDAC6 IN LYMPHOID MALIGNANCIES, A NEW POTENTIAL THERAPEUTIC COMBINATION

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Introduction: Momelotinib is a potent inhibitor of JAK1/JAK2 that demonstrated efficacy in patients with primary and secondary myelofibrosis. Citarinostat, a second generation HDAC6 selective inhibitor, is a well-tolerated compound compared with nonselective HDAC inhibitors, with reduced potency against Class I HDACs while retaining anticancer effectiveness.

Methods: Momelotinib and citarinostat alone and in combination were tested in 12 lymphoid cell lines: WSUNHL, RL, Karpas422 (FL), Granta519, Jeko1 (MCL), Hut-78 (CTCL), Karpas299 (ALCL), L540, L1236 (LH), U266, RPMI8266 (MM) and MEC1 (CLL). Synergistic interaction

was evaluated using the Chou-Talalay method. Annexin V staining, ROS generation and cell cycle were determined by flow cytometry. ATP levels and Mitochondrial Membrane Potential ($\Delta\Psi m$) were evaluated by fluorometric assay. Lactate levels and Cyt-C were evaluated by colorimetric assay. Activity of caspases-8,-9 and 3 was measured using colorimetric assay. ER stress and apoptosis-related proteins and JAK2/STAT3 were detected by western blotting. Clonogenic survival was studied with the methylcellulose clonogenic assay. Co-coltures with bone marrow stromal cells were also performed.

Results: The combination of momelotinib (1 μM) and citarinostat (4 μM) at 24h showed a synergistic effect in WSUNHL, RL, Karpas422, Jeko1, Hut78, Karpas299, L540, RPMI8226 and U266 cells with CI values < 1 and antagonist effect in L1236, Granta519 and Mec1 cells with CI > 1. We studied seven lymphoid cell lines (WSUNHL, RL, Karpas422, Jeko1, L-540) which were particularly sensitive to the drug combination and two cell lines (L-1236, Granta-519) that showed an IC > 1. Drug combination exhibited a strong cytotoxicity, evidenced by reduction of mitochondrial depolarization, depletion of ATP and lactate levels and Cyt-C release from the mitochondria but also by increase in cellular apoptotic index and reactive oxygen species (ROS) levels, leading to arrest in the sub-G0/G1 phase of the cell cycle. Apoptosis induced by the drug combination was exerted via the mitochondrial apoptotic pathway as demonstrated by upregulation of caspase-9 that was especially evident in WSU-NHL and Karpas422 with a fold increase of 3.2 and 3.8. The extrinsic apoptotic pathway was active in Karpas422 and Jeko1 cells as evaluated by upregulation of caspase-8 but not in WSU-NHL, RL and L540. The apoptosis was associated with activation of caspase-3, PARP and with increased hallmarks of ER stress and was mediated by the increased expression of the pro-apoptotic proteins Bad, Bax and Bim and downregulation of Bcl2, Bcl-xL and Mcl-1. Drug combination reduced clonogenic survival and suppressed cell viability of lymphoid cells when co-cultured with bone marrow mesenchymal stromal cells targeting JAK2/STAT3 pathway and confirming acetylation of acetyl-α tubulin.

Conclusion. Due to the good toxicity profile and the oral administration, the combination has the potential for a completely orally therapeutic approach. The study is ongoing and further investigation is required.

PO106

LENALIDOMIDE (LEN) UPREGULATES PROGRAMMED DEATH-1 (PD-1) PATHWAY IN ACTIVATED LOW-GRADE LYMPHOMA CELLS

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Introduction: PD1 is expressed on T cells, B cells, and other immune effector cells of HL and NHL. PD1/PDL1 crosslinking induces negative signals to T cell, provides a T-cell exhaustion state and, provokes neoplastic tolerance. The tumor microenvironment (TME) is essential for proliferation and survival of lymphoma cells and in resisting the effects of chemotherapy. Interrupting the signaling pathways mediated by cells or humoral factors might enhance the effects of chemotherapy and suggests that the TME is a target for therapy. LEN is an oral IMID with direct antineoplastic activity and immunologic effects, including T cell– and NK cell–mediated cytotoxicity in experimental models. Preclinical findings indicate that combination of IMIDs with immune checkpoint inhibitors may promote therapeutic synergy and long-term antitumor immunity to improve clinical outcome. We characterized PD1, PDL1/PDL2 phenotypes in peripheral neoplastic CD19+ lymphocytes and Tcell subsets in low-grade B-cell lymphoma primary cells; we evaluated the role of the TME in supporting the PD1 axis; and determined whether LEN influences PD1 or PDLs expression.

Methods: Cell samples from PBMCs of low grade lymphoma patients in the leukemic phase were used to determine PD1, PDL1, PDL2 phenotype by flow-cytometry (FC). Autologous activated T-cells (AAT) were obtained by *in vitro* coculture of patient T-cells with anti-CD3/CD28 beads, rIL2, and with PBMCs. In selected experiments LEN (Celgene) was added to cell cultures.

Results: 28 cases of lymphoma were evaluated for PD1 and PDLs expression on malignant B- and T-cells. PDL1 expression was practically undetectable, while PD1 and PDL2 were similarly expressed on B-cells. Significantly higher PD1 compared with very low levels of ligand expression were detected in CD4+ and CD8+ cells. Co-culture of lymphoma cells with AAT cells showed consistent formation of B/T-cell clusters. PD1 and PDLs expression significantly increased in AAT coculture on B-cells. PD1 expression on CD3+ cells was unaffected by AAT, although the PDL1/PDL2 expression increased significantly. PD1 expression increased significantly only in CD4+ cells. Lymphoma-AAT co-culture experiments (n=4) indicated that LEN (0.5/1uM) did not negatively influence the formation of AAT clusters. After 48h of coculture, the expression of CD19+CD5-PDL1+ cells increased in 4/4 cases following LEN treatment while, PDL2 expression remained constant. LEN increased CD3+PD1+ expression, while the expression of PDLs remained unaffected. Evaluation of activated T-cell subsets showed similar results, with the exception of stronger induction of PD1 and PDL1 expression by LEN in CD8+ cells.

Conclusions: The PD1/PDLs-axis is potentially involved in lymphoma immune responses. LEN treatment induces the expression of PD1 in CD8+ and CD4+ cells and may contribute to reactivate PD1 signaling under treatment. The PD1 pathway may be potentially targeted to overcome both the intrinsic and LEN-induced exhaustion phenotype. Celgene grant to FM.

PO107

ACTIVATION OF GPER SIGNALING AS A POTENTIAL THERAPEUTIC STRATEGY AGAINST WALDENSTRÖM MACROGLOBULINEMIA

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Introduction: Estrogens have been described as regulators of differentiation, proliferation and survival of normal and malignant B cells. However, the impact of estrogen receptors' signaling on Waldenström Macroglobulinemia (WM) biology remains unexplored. By means of pharmacologic agonists and antagonists, we investigated the druggability of estrogen receptors, namely the classical estrogen receptor ERbeta and the alternative 7-transmembrane G-protein coupled estrogen receptor GPER. While ERbeta pharmacological modulation did not elicit any

effect, GPER agonists reduced WM cell survival. This finding prompted us to elucidate GPER expression and function in WM.

Methods: mRNA levels were assessed by qRT-PCR; protein expression was evaluated by WB or IHC. Cell viability and apoptosis were analyzed by Cell Titer Glo, Annnexin V/7AAD and antibody arrays. Cell migration was assessed by transwell assay, clonogenicity by methylcellulose cultures. Subcutaneous xenografts in SCID mice were used to evaluate *in vivo* effects of GPER agonists.

Results: We detected GPER expression at mRNA and protein levels in 2 WM and 3 lymphoma cell lines. By interrogating publicly available microarray datasets, we found GPER significantly upregulated in WM as compared to normal B cells. IHC analysis showed overexpression of GPER protein in lymph node biopsies from newly diagnosed WM patients (n=6) as compared to healthy lymph nodes (n=3). To unravel GPER-mediated signaling, we took advantage of the selective GPER agonist G-1 ((±) -1-[(3aR*,4S*,9bS*) -4-(6-Bromo-1,3-benzodioxol-5-yl) -3a,4,5,9b-tetrahydro-3H cyclopenta [c]quinolin-8-yl] ethanone). At low micromolar concentration, G-1 inhibited the survival of WM (n=2), lymphoma cell lines (n=3), and primary WM cells (n=3), while it did not affect healthy CD19+ B cells (n=3); moreover, G-1 overcame the protective BM stromal cells in co-culture experiments, reduced WM cell migration and clonogenicity, and synergistically enhanced bortezomib and ibrutinib anti-tumor activity. Conversely, GPER antagonists G-36 and G-15 slightly increased cell proliferation. GPER activation increased cells in G2/M phase and triggered apoptosis. Importantly, intraperitoneal injection of G-1 (2 mg/kg) significantly reduced the growth of subcutaneous BCWM-1 xenografts in mice. To shed light on the molecular sequelae underlying GPER activation, we carried out gene expression profiling and GSEA analysis of G-1-treated BCWM-1 cells. Intriguingly, the p53 signaling pathway was strongly induced by GPER activation, and upregulation of p53 and its target genes (p21, Bax, Bad, PUMA) was confirmed in both BCWM-1 and primary G-1 treated WM cells; moreover, G-1 combination with bortezomib or ibrutinib led to stronger increase in p53 and p21 levels.

Conclusions: Altogether, our results indicate that GPER is expressed by WM cells, and its activation triggers potent anti-tumor activity, providing the framework for future clinical investigation of GPER-agonists.

PO108

DIFFERENCES OF PD-1 EXPRESSION ON MATURE B-CELL NEOPLASMS

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Introduction: The immune checkpoint has emerged as an important target in cancer immunotherapy. The interaction between the programmed cell death protein 1 (PD-1) on tumor-infiltrating T cells and its ligand PD-L1 on tumor cells has been addressed in many studies. PD-1 is expressed as well during normal B cell development. There are only few data on PD-1 expression on neoplastic B cells. Using the liquid biopsy technique, we characterized expression of PD-1 on mature B-cell neoplasms.

Methods: We included 71 patients with mature B cell neoplasms: 31 chronic lymphocytic leukemia/small lymphocytic lymphomas (CLL/SLL), 17 follicular lymphomas (FL), 13 diffuse large B-cell lymphomas (DLBCL) (5 germinal center B (GCB)-cell type, 8 non-GCB type), 6 mantle cell lymphomas (MCL), 4 primary mediastinal lymphomas and 4 patients with non-malignant lymph node biopsies. Flow-cytometric assessment was performed on 41 lymph node suspensions, 29 peripheral blood samples and 5 bone marrow samples. Lymph node suspensions were prepared by mechanical disaggregation of solid tissue using the Medimachine system (BD Biosciences). PD-1 (CD279, clone EH1 2.1, BD Biosciences) expression was assessed on neoplastic B-cells. Data were acquired on BD FACSCantoII flow cytometer (BD Biosciences) and analyzed using BD FACSDiva software (BD Biosciences). Data were expressed as percentage of expressing cells and median fluorescence intensity (MFI).

Results: PD-1 expression was low (<2%, MFI<150) on B cells from non-malignant lymph nodes. A significantly higher proportion of neoplastic B cells expressed PD-1, with important differences according to histology (p<0.001). The highest PD-1 expression was observed among CLL/SLL (median, 34.3%, range: 4.5% to 98.1%), and the lowest in FL (median, 1.1%, range: 0.2% to 5.8%). Among patients with DLBCL, PD-1 expression appeared to be lower in patients with GCB-type DLBCL (median 3.8% versus 31% in non-GCB, p=0.06). The median PD-1 expression in MCL was 16.6% and 3.8% in PMBCL. Using a cut of 10% positive cells, 84% (26/31) of CLL/SLL, 67% (4/7) of MCL, 75% (6/8) of non-GCB type DLBCL, but only 20% (1/5) of GCB-type DLBCL (1/5), 25% (1/4) of PMBCL, and 0% (0/17) of FL were considered as PD-1 positive. The PD-1 expression pattern was confirmed also when analyzing MFI values. PD-1 was strongly expressed on tumor-infiltrating T cells from lymph node biopsies of all lymphoma types. The median MFI for PD-1 on CD3+ cells from non-malignant biopsies was 464, while it was 3995 for FL and 3965 for DLBCL without difference between GCB and non-GCB.

Conclusions: PD-1 is expressed on a significant proportion of neoplastic B cells in lymphomas not deriving from the germinal center (CLL/SLL, MCL, non-GCB DLBCL), while its expression is low on GC-derived lymphomas, as FL and GCB-type DLBCL. We observed strong PD-1 expression on tumor-infiltrating T cells also in GC-derived lymphomas.

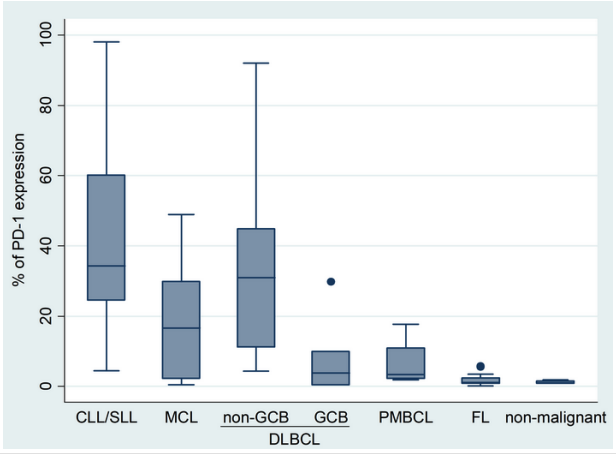

Figure 1. GRAPH PD-1 B NEOPLASM.

PO109

CD30+ REGULATORY T-CELLS ARE REDUCED IN RELAPSED/REFRACTORY HODGKIN LYMPHOMA PATIENTS ACHIEVING COMPLETE REMISSION AFTER EXPOSURE TO SINGLE-AGENT BRENTUXIMAB VEDOTIN: A PROSPECTIVE SINGLE-CENTER STUDY

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Background: Hodgkin Lymphoma (HL) is associated to deep microenvironment re-shaping and myeloid dysfunction. Our previous work showed that myeloid-derived suppressor cells (MDSC) are increased in HL patients' peripheral blood and predictors of outcome at baseline. Patients who have relapsed/refractory (R/R) disease after frontline therapy have dismal outcome and typically undergo salvage chemotherapy followed by high-dose conditioning and autologous stem cell transplant

(ASCT). Brentuximab Vedotin (BV) is a novel CD30-directed ADC approved for the treatment of R/R HL. The conjugation of a CD30-specific monoclonal antibody to a potent microtubule-disrupting agent [monomethyl auristatin E (MMAE)] via a stable linker allows the targeted delivery of high doses of the cytotoxic drug to CD30-expressing cells, leading to high therapeutic efficacy. Besides targeting CD30+ lymphocytes through direct binding, an important mechanism of action of BV may be the bystander effect of MMAE, which diffuses out of the CD30+ lymphocytes into their microenvironment.

Aim: Only limited data are available on the role of Brentuximab Vedotin (BV) as single agent in transplant-naive R/R patients and its off-target effects on immune system. Thus, we prospectively analyzed regulatory T-cells (CD4+CD25highCD127low/+T-reg) and myeloid-derived suppressor cells subpopulations (CD11b+CD33+CD15+CD14-HLA-DR- G-MDSC, CD11b+CD33+CD15-CD14+HLA-DR- mo-MDSC) in peripheral blood by flow cytometry in 15 patients with primary R/R HL who had not received prior high-dose chemotherapy and ASCT before, during and after treatment with BV as single agent in a named patient program.

Results: After median follow-up of 32.2 months, all patients except two were alive (87%). Overall survival at 36 months OS was 80% and PFS was 53.3%. Four cycles of BV exposure reduced the absolute number of three subtypes of myeloid-derived suppressor cells (CD34+MDSC, mo-MDSC, G-MDSC, $p<0.001$). The MDSC marker arginase-1 (s-Arg-1) was increased at baseline, reduced after two BV cycles, achieving levels lower than healthy subjects upon four BV cycles ($p=0.02$). However, CD30 expression was very low on MDSC surface, as sorted by both peripheral blood and bone marrow, excluding a direct toxic effect of BV against myeloid precursors. The percentage of T-reg cells in peripheral blood of R/R HL patients was higher than healthy controls ($p=0.0001$) and reduced after treatment ($p=0.02$). CD30+CD4+CD25highCD127low/+T-reg (CD30+ T-reg) were progressively reduced upon treatment in both bone marrow and peripheral blood, with a progressive reduction in expression of CD30 ($p=0.01$).

Conclusions: BV was safe and well-tolerated in R/R HL patients. Our data suggest that the effect of BV is not limited to CD30+ lymphoma cells but other cellular components of the microenvironment could be affected by BV treatment

PO110

REVERSAL OF ACQUIRED GLANZMANN THROMBASTHENIA AFTER RITUXIMAB TREATMENT IN A PATIENT WITH CUTANEOUS B-CELL LYMPHOMA

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Introduction: Platelet dysfunction is a rare acquired hemorrhagic disorder, described often in association with lymphoproliferative and other autoimmune disorders. We describe the case of a 68-year-old woman with cutaneous B-cell lymphoma coming to our attention in December 2017 for a moderate mucocutaneous hemorrhagic picture. Family history was negative for coagulopathies. In 2011 diagnosis of cutaneous B-cell lymphoma, treated surgically and with radiotherapy. June 2012 episode of idiopathic thrombocytopenic purpura resolved with steroid therapy. February 2017 recurrence of cutaneous lymphoma with amyloid deposits (no therapy). April 2017 appearance of diffuse spontaneous ecchymosis and, from August 2017, episodes of epistaxis, gum bleeding and an episode of conjunctival hemorrhage.

Methods: Platelet aggregation was studied in platelet rich plasma (PRP) by light transmission aggregometry (LTA, Chrono-log), and in whole blood by the Multiplate and PFA-100 systems. Platelet glycoproteins (GP) were measured by flow cytometry. Anti-platelet antibodies were determined in plasma by ELISA (PakPlus, Immucor)

Results: Platelet count, routine coagulation tests and screening for vWF disease were normal. Closure times at the PFA100 were pathological (>300s). Platelet aggregation in whole blood was completely absent with all stimuli. Analysis of platelet GP in whole blood showed normal GPIb GPIIb/IIIa and GPIIIa expression, although the mean fluorescence intensity of GPIIbIIIa was low. The expression of P-selectin after stimulation was normal. In a subsequent visit, LTA study showed that the patient's platelets did not respond to any of the agonists except to ristocetin, which, however, induced a reversible agglutination. In mixture experiments, patient's plasma significantly reduced aggregation of control platelets in LTA, as well as binding of an anti-GPIIb/IIIa MoAb on the control platelets, without, however, modifying the binding of anti-GPIb and anti-GPIIIa. These data suggested the presence of a platelet inhibitor. Antiplatelet antibodies measurement revealed high titer of anti-GPIIbIIa. The patient then started immunosuppressive therapy with corticosteroids (1 mg/kg/day). At 10-day and 16-day follow-up, platelet function monitoring by Multiplate showed partial recovery of platelet activity, although closure times at PFA 100 were still abnormal. The patient clinically improved. Rituximab was then started, and after 3 cycles, platelet function assessment was found normal by PFA100 and Multiple. LTA was normal at all stimuli, except for epinephrine.

Conclusions: The aggregation profile in our patient suggests the presence of a platelet function inhibitor that produces a defect similar to Glanzmann thrombasthenia, without affecting platelet count. The inhibitor was identified as an anti-GPIIbIIa autoantibody. Although it may be a rare event, one should be aware of the acquired platelet dysfunction as a cause of an unexpected primary hemostatic disorder in patients with lymphoma.

PO111

THE NEUTROPHIL PD-L1 AND LYMPHOCYTE PD-1 AXIS IN THE PERIPHERAL BLOOD IN CLASSICAL HODGKIN LYMPHOMA

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Introduction: High levels of tumor-infiltrating neutrophils have been shown to be associated with advanced disease and poor clinical outcome in patients with cancer. The ratio between the absolute neutrophil count (ANC) and the absolute lymphocyte count (ALC), the neutrophil-lymphocyte ratio (N/L ratio) has been reported as predictor of progression-free survival (PFS) in cancer patients including classical Hodgkin lymphoma (cHL). We reasoned that the immune checkpoint axis between the Programmed cell death protein 1 (PD-1) and its ligand PD-L1 could be involved in the neutrophil-lymphocyte interaction in HL. We evaluated N/L ratio and PD-L1/ PD-1 axis in the peripheral blood at diagnosis of cHL using flow cytometry and RT-PCR and analyze for associations with clinical characteristics and outcome.

Methods: A total of 408 patients diagnosed with cHL between 1999 and 2017 were included to analyze for the prognostic impact of N/L ratio. Expression of PD-L1 and PD-1 was studied using RT-PCR in buffy-coats of 82 patients, and in cell fractions from peripheral blood of normal donors (n=4) and patients (n=24) separated using magnetic beads. PD1 (CD279,BD Biosciences) and PD-L1 (CD274, Beckman Coulter) was prospectively analyzed by flow cytometry in a group of 14 patients and 5 normal volunteers. PFS was evaluated with logrank and Cox regression models, stratified for the type of treatment.

Results: We first confirmed the prognostic significance of the N/L ratio in our case series of 408 patients. A high N/L ratio (≥ 6) was associated with poor PFS ($p=0.003$) in univariate analysis and retained its significance in a multivariate analysis including the most powerful predictor in HL, interim PET as independent prognosticator (HR 2.4; 95% C.I. 1.3-4.2, $p= 0.004$). We then analyzed PD-L1 and PD-1 expression using flow cytometry and RT-PCR. Both methodologies showed that PD-L1 expression was highest in neutrophils and PD-1 expression was highest in the T cell fraction. Furthermore we found a correlation

between expression of PD-L1 on neutrophils and PD-1 on T cells (r=0.6 p=0.008). Both methodologies also showed a significantly increased expression of PD-L1 on neutrophils from patients when compared to controls, with particular high PD-L1 expression on neutrophils in patients with Stage IV. PD-L1 expression on neutrophils did not correlate with neutrophil count but with the N/L ratio (p=0.004). PD-L1 expression on neutrophils was associated with a poor PFS (p<0.001), that was maintained when including also the N/L ratio in a multivariate analysis (HR 3.1, 95% C.I., 1.3-9.1, p=0.03).

Conclusions: Increased expression of the immune checkpoint protein PD-L1 on neutrophils in patients with HL at diagnosis was associated with increased disease activity and a change in the balance between neutrophils and lymphocytes, suggesting an inflammatory and immunosuppressive reaction. The identification of a druggable target of prognostic relevance at diagnosis might help to develop personalized therapeutic strategies.

Molecular Hematology

PO112

THE INTEGRATION OF MULTIPLE GENETIC ANALYSIS IMPROVES THE DIAGNOSTIC PROCESS AND PROVIDES A BETTER RISK-STRATIFICATION IN ACUTE MYELOID LEUKEMIA

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Introduction: Acute Myeloid Leukemia (AML) is generally characterized by recurrent genetic aberrations as chromosomal translocations, gene mutations and copy number variations (CNVs). Molecular testing for AML-related genetic markers is strongly recommended by all international guidelines as an essential step for proper diagnosis and subsequent monitoring of AML. Moreover, genetic assessment proved useful for the correct risk-category allocation of the patients. In the absence of informative genetic lesions the therapeutic decision-making process might become challenging. We report a case of AML in a young adult in which standard genetic analysis, performed at disease onset according to European LeukemiaNet (ELN), showed no alterations.

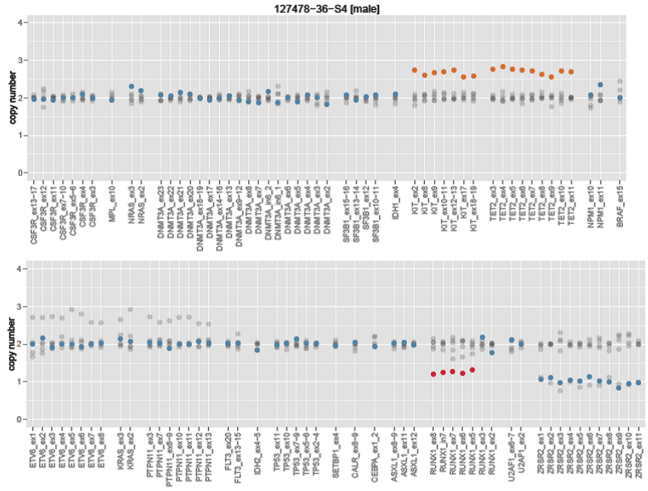

Fig. 1: NGS-derived CNVs detection: the duplication involves all the analyzed exons of *KIT* and *TET2* genes (orange dots), while the deletion of *RUNX1* gene (red dots) does not affect exons 2 and 3.

Figure 1.

Methods: We analyzed the diagnostic bone marrow sample of a young AML patient by conventional cytogenetic analysis, fluorescence *in situ* hybridization (FISH) and standard molecular assays as qualitative PCR, RT-PCR, enzymatic digestion and fragments analysis. Then, we performed a capture-based Next Generation Sequencing (NGS) assay, covering coding regions and splicing junction of 30 genes associated to myeloid malignancies. Libraries were prepared starting from 200 ng of gDNA using the Myeloid Solution kit (SOPHiA GENETICS) and paired-end sequenced on the MiniSeq platform (Illumina). NGS data were analyzed by the SOPHiA DDM® tool. NGS-derived CNVs results were validated by Comparative Genomic Hybridization (CGH) array (4x180K, Agilent) using 500 ng of gDNA.

Results: Cytogenetic analysis performed at diagnosis was unsuccessful due to the lack of metaphases and FISH assay for TP53 and KMT2A provided normal Results: Molecular evaluation of recurrent fusion genes as RUNX1-RUNX1T1, CBFB-MYH11, BCR-ABL1 proved negative

and no mutations were identified in NPM1, CEBPA, and FLT3 genes. The capture-based NGS assay did not revealed any pathogenic Single Nucleotide Variant (SNV) or Insertion/deletion (Indel) in the 30 genes included in the panel, but suggested a few CNVs: a duplication of KIT and TET2 genes and a deletion of RUNX1 from exon 5 to 8 (Figure 1). CGH array confirmed a trisomy of chromosome 4, where KIT and TET2 genes are mapped, and a partial deletion of chromosome 21 containing a portion of the RUNX1 gene. In addition, a partial tetrasomy of chromosome 13 and a partial deletion of chromosome 17, regions not covered by the NGS panel, were identified by CGH array. The risk-category of the patient was revised following the ELN guidelines and the treatment was modified accordingly.

Conclusions: The laboratory evaluation of leukemia genetic profile is complex and has evolved significantly with the incorporation of advanced techniques. The combination of multiple innovative genetic approaches could help in identifying prognostic markers leading to a proper risk category stratification and a better patient management, especially in those cases in which standard approaches fail.

PO113

ALTERED EXPRESSION OF JAK-STAT PATHWAY AS POSSIBLE PREDICTIVE MARKER IN PATIENTS AFFECTED BY CHRONIC MYELOID LEUKEMIA RECEIVING TYROSIN KINASE INHIBITORS (TKIS)

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The JAK-STAT pathway is involved in the transduction of signals mediated by cytokines, interferons and growth factors with consequent support of neoplastic cell growth and invasion in many types of cancer. Additional implications in inflammation and immunity in the tumor microenvironment have been recently recognized, because this pathway seems to sustain the stem cell maintenance. Moreover, persistent STAT3 activation would confer resistance to therapy with tyrosine kinase inhibitors in CML by controlling the leukemia stem cell self-renewal and favoring its hiding in the bone marrow niche (Groner, 2017). Inhibition of this pathway might represent a potential way to ameliorate the molecular response in warning or failed CML patients or to sustain deep responses in cases tempting the discontinuation of therapy. Our purpose was the evaluation of some BCR/ABL1-independent molecular predictive markers of response in CML patients. Thus, we analyzed the expression of 86 genes belonging to the JAK-STAT pathway in 10 cases assessed at diagnosis and after 6 months of therapy with TKIs. We quantitated the expression level of 86 JAK-STAT genes by RT-qPCR (PrimePCR SYBR® Green assay, Biorad©, Milan, Italy) at baseline and after 6 months of therapy. According to ELN guidelines, after six months of treatment 9 patients were in optimal response and 1 was in failure. Indeed, 79 genes resulted up-regulated, while only 7 were down-expressed. We correlated the gene expression results with the achievement of MR3. At 6 months of treatment, we identified correlation of MR3 with up-regulation of LRG1 (p=0.030), a gene belonging to the leucine-rich repeat (LRR) family that is overexpressed during the granulocyte differentiation, and down-regulation of IL2RA (p=0.030) and MPL (p=0.029).IL2RA is involved in the LSC growth and MPL is linked to persistent activation of JAK-STAT.Moreover, the up-regulation in responsive patients also involved the immunity signaling linked to interferon (IFN) receptors complex, and induced interferon-related factors with anti-proliferative and pro-apoptotic effects, such as CSF1R, IRF1, IRF9, and ISG15.The increased expression of GATA3, SOCS3, JAK3 is involved in NK and T cell recruitment as immunology protection in responsive patients. Finally, in responsive cases we observed a significant up-regulation of: OSM, involved in bone remodelling and reduction of fibrosis damaging the LSC survival in the niche and IFN receptor type 1 that is usually expressed in stromal cells as a protection factor against cancer progression. In this work, we demonstrated that the JAK-STAT pathway is really implicated in the resistance to TKIs and, on the other hand, that the de-regulation of some genes of this family might be related to the achievement of better molecular responses. This observation could have a practical clinical output, suggesting the effectiveness of the combination of JAK-STAT inhibitors (ruxolitinib or methotrexate) with TKIs in resistant CML patients.

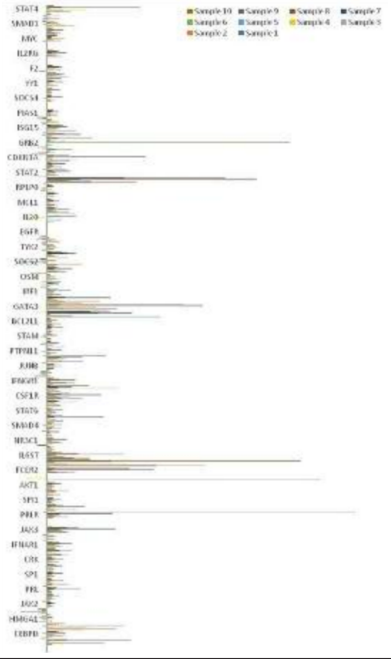

Figure 1.

PO114

WNT PATHWAY IS INVOLVED IN BCR-ABL1-INDEPENDENT RESISTANCE TO TKIS IN CHRONIC MYELOID LEUKEMIA PATIENTS

S. Grassi, S. Palumbo, V. Mariotti, E. Ciabatti, F. Guerrini, C. Baratè, M. Petrini, S. Pellegrini, S. Galimberti

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Background: After the introduction in the clinical practice of TKIs, the overall survival of CML patients is really improved, but several mechanisms of resistance have been reported. In addition to BCR-ABL1-related mechanisms, the persistence of the leukemic stem cell in the BM niche is a very relevant problem. The hypoxia and the presence of immunosuppressive cells in the microenvironment are well-known mechanisms that sustain the LSC; nevertheless, an increasing interest is today put also into the WNT/Beta-catenin pathway, that is necessary to self-renewal of normal cells also, but whose deregulation causes leukemogenesis and progression in several types of cancers (Zhao, 2007).

Aims:we decided to assess the expression of 86 genes of the beta-catenin/WNT pathway at diagnosis and after 6 months of treatment with TKIs in a cohort of 10 patients with different responses to treatment. Methods: Buffy coats obtained from peripheral blood samples of 10 patients (7 receiving imatinib, 2 nilotinib, and 1 dasatinib) have been used for the total RNA extraction. We used RQ-PCR for measuring the expression of 86 genes from the WNT pathway (PrimePCR pathway kit, Biorad, Milan, Italy) at diagnosis and after 6 months of therapy. Expression values were calculated by the Vandesompele method using four housekeeping genes. Data has been evaluated with "Gene Study" PrimePCR analysis software (Biorad).

Results: In our series of patients 5 achieved an optimal response and 5 were no responders, according to the ELN guidelines. Interestingly after 6 months of treatment, we observed a de-regulation of 36 genes. Down-expression occured in 14% of genes, while 79% of genes were up-regulated. When we compared the change of expression with the quality of response to TKIs, a differential expression between patients

with or without an optimal response was observed: in the cases without optimal response, we found as up-regulated: 1) FZD7, already known to be responsible for the protection of leukemic CML cell, proliferation and drug resistance in K562 cells; 2) WNT6, that predicts unfavorable survival in solid cancer and whose expression is inversely correlated to the response to ECF (Epi, cisplatin, 5-fluorouracil) chemotherapy in human gastric cancer cells; 3)WISP1, with anti-apoptotic activity, associated to poor prognosis and advanced stage in glioblastoma. On the other hand, the most frequently down-regulated gene was CSNK1A1, whose aploinsufficiency has been shown to result in a more probable transformation of MDS in AML and to induce proliferation, invasion and metastasis in MM, DLBCL and AML.

Conclusions:With this experiments of gene expression profiling we demonstrated, even if a small series of CML patients, that the beta-catenin /WNT pathway could be relevant in the conditioning the response to TKI. Obviously, the analysis of a larger number of patients will improve the biological suggestions coming from these preliminary data.

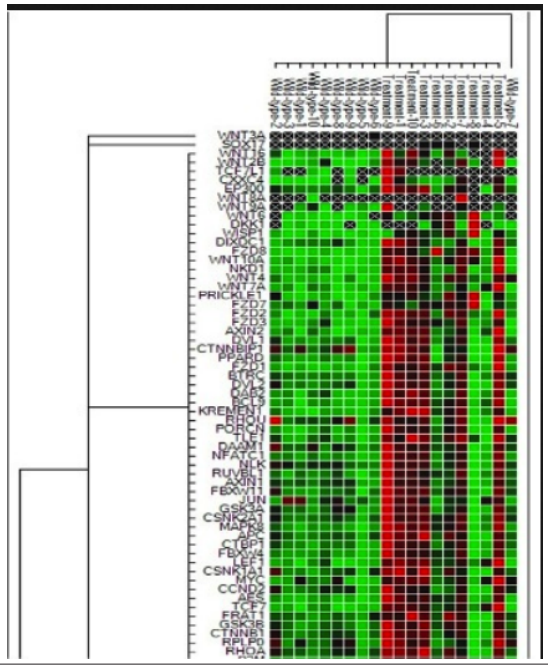

Figure 1.

PO115

BMI1 IS CO-EXPRESSED WITH BCR-ABL1: THE EXPERIENCE OF THE CONFOCAL MICROSCOPE

S. Grassi, L. Mattii, F. Perutelli, G. Del Genio, M. I. Ferreri, C. Giuliani, A. Di Paolo, E. Abruzzese, M. Petrini, C. Baratè, S. Galimberti

Dipartimento di Biotecnologie Mediche, Università di Siena; Dipartimento di Medicina Clinica e Sperimentale, Università di Pisa; Dipartimento di Medicina di Laboratorio, SOD Citogenetica, AOUP; UOC Ematologia, Ospedale S. Eugenio, Roma, ITALY

Background: The introduction of Tyrosine kinase inhibitors (TKIs) improved overall survival in CML patients, today comparable to that of age- and sex-matched general population (Gunnarson et al. 2016). Nevertheless, a third of patients do not achieve deep molecular responses and has to suspend treatment (Hochhaus, 2009). This phenomenon can occur for the appearance of ABL1 mutations, but also numerous BCR/ABL1-independent mechanisms seem to be at the basis of resistance to TKIs. We previously demonstrated the correlation between some polycombs genes and prognosis, with the BMI1 oncogene high expression resulting to negatively condition the achievement of cytogenetic and molecular response (Crea, 2015). In the other hand, also the survival of leukemic stem cell (LSC) in the BM niche may cause resistance to TKIs. LSCs cells exhibit CD34+/CD38- phenotype, but recently the cytokine-targeting surface enzyme dipeptidyl- peptidase IV (CD26) has been added as phenotypic marker. We hypothesized that BMI1 could be expressed by immature cells and thereby able to cause a ABL1-independent resistance to TKIs. Therefore, we decide to use the laser scanning confocal microscopy to perform co-expression experiments.

Methods: Triple-immunofluorescence analysis was performed on buffy coat smears from 10 CML cases at diagnosis. Smears were treated with -MαBCR/ABL (1:300, Thermo Fisher, Rockford, IL, USA) , -RαBMI-1 (1:100, Thermo Fisher), and -GαCD26 (1:100, R&D system, Minneapolis, MN, USA) antibodies. Nuclei were stained with DAPI. The samples were observed at 20x, 43x or 63x magnification by a confocal laser scanning microscope (TC SSP8 Leica Microsystems, Mannheim, Germany) using a 488-nm, 561-nm and 642-nm excitation wavelength lasers. Negative controls for secondary antibodies were performed omitting primary antibodies. As further negative controls, the same reactions were performed on samples from patients affected by acute leukemia (AML), follicular lymphoma (FL), and essential thrombocytemia (ET).

Results: As shown in the figure, the analysis of the immunofluorescence pattern clearly showed the co-expression of BCR/ABL1 (green fluorescence), BMI1 (red) and CD26 (grey) proteins. Moreover, the tridimensional reconstruction documented for all these proteins a cytoplasmic localization. Moreover, we found the expression of BMI1 in AML, FL, and ET; nevertheless, as expected, this protein was not co-expressed with BCR-ABL1, nor with CD26, thus confirming the specificity of the finding.

Conclusion: Our work clearly demonstrated that the BMI1 protein co-localizes with BCR-ABL1 and CD26. This finding opens new interesting perspectives: in CML, BMI1 is an additional marker of the LSC and could be responsible for ABL1-independent resistance to TKIs. Thus, we can hypothesize that BMI1 could represent a new target for a patient-oriented treatment alternative to TKIs in CML resistant cases.

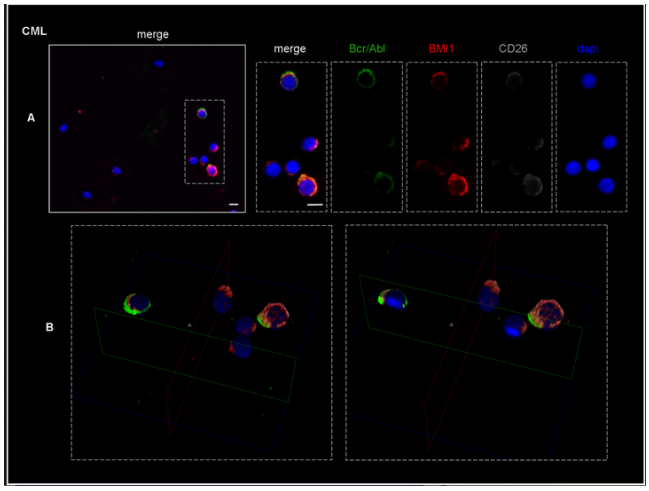

Figure 1.

PO116

A SIMPLE PCR-BASED TOOL FOR THE ASSESSMENT OF SOMATIC MUTATIONS IN ACUTE MYELOID LEUKEMIA AND ITS PROGNOSTIC POWER

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Background: Cytogenetic and molecular parameters are today relevant in the classification and prognostication of acute myeloid leukemia (AML). Among the molecular techniques, the NGS allows an accurate characterization, but optimization is often complicated and it is not avail-

able everywhere and conventional PCR could be a surrogate for the mutational assessment.

Patients and Methods: we used a custom-made version of the qBiomarker Somatic Mutation PCR Arrays (Qiagen, Milan, Italy) for assessing by simple real-time PCR mutations of ASXL1, TET2, IDH1, IDH2, NRAS, WT1, c-KIT, RUNX1, FLT3, NPM1, and DNMT3A in 38 AML patients receiving 3+7 regimen or demethylating agents before eventual allogeneic transplantation (in 8 cases). Then, molecular data were imputed on the Sanger website (algorythm that estimates OS probability).

Results: We found that 81% of cases were mutated, with a median of 2 mutations/patient: 60% showed c-KIT mutations, 26% mutations of N-RAS, 16% of IDH2, NPM1, or RUNX1, 13% of FLT3, 10% of IDH1; 8% of WT1 or DNMT3A, and 3% of TET2 or ASXL1. DNMT3A was more frequently mutated in older patients. After induction, two third of patients achieved a response, with 48% of CRs and 18% of PRs; presence of mutations significantly impacted on the response rate (100% for unmutated vs 42% for mutated patients). At 2 years, 30% of patients were still alive; sex, age, WHO classification did not significantly condition the OS. On the contrary, the cytogenetic score predicted the outcome, with a median of 8 months for patients with adverse cytogenetics, 12 months for those with intermediate risk, and a median not reached at 24 months for cases with favorable karyotype (p=0.001). About mutations, their presence/absence did impact on OS, but only in cases with a favorable cytogenetics, with a median OS of 23 months for cases with low vs 6 months for cases with high mutational burden (>3 mutations) (p=0.03). A trend to a longer OS was observed for patients with low WT1 expression. In the subjects with favorable or intermediate risk score, to have 3 or more than 3 mutations represented a poor prognostic factor also on PFS, with a median of 18 months for cases with low vs 4 months for cases with high mutational burden (p=0.04). Finally, we imputed clinical, cytogenetic and molecular features in the Sanger website (http://cancer.sanger.ac.uk/aml-multistage/) thus calculating the probability of death after relapse and of being alive free of disease at 3 years. In the subgroup of the mutated cases we observed a significant change of these probabilities in 86% of cases (in 2/3 with impairment of prognosis).

Conclusions: In summary, the somatic mutational assessment also by PCR instead of NGS would be useful for a more correct stratification of AML patients.

PO117

CLINICAL VALIDATION OF MYELOID SOLUTION PANEL ON ION TORRENT S5 PLATFOR

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Introduction: The genomic landscape of myeloid neoplasms has changed during last decade and the genetic aberrations can be grouped according to their functional role (Papaemmanuil E, NEJM, 2016). Several Next Generation Sequencing panels are available to detect gene mutations and to guide patient management. Myeloid Solution (SOPHiA GENETICS) is a CE-IVD marked gene panel for sequencing myeloid leukemia samples on Illumina sequencers. We evaluated its applicability also on Ion Torrent S5 (ThermoFisher Scientific, MA) sequencer.

Methods: 23 myeloid neoplasm samples were sequenced and analysed on MiSeq (Illumina, CA) and/or Ion Torrent S5 sequencers. Four runs were performed: the first on MiSeq using a V3 cartridge (24 samples), the others on Ion Torrent S5 using 530 sequencing chip. The enrichment and emulsion steps were performed by One Touch 2 (OT2) and Enrichment System for the first and second S5 runs (12 samples); Chef System was used for the third S5 run (8 samples). Intra/run replicates were performed. DNA was extracted using Maxwell 16LEV Blood kit (Promega) from peripheral blood or bone marrow mononuclear cells after isolation on Ficoll gradient. SG001 and HD701 cell-line samples were used as positive controls. Amplicon libraries were prepared by Myeloid Solution sequencing panel starting from 200ng (Illumina) or 500ng (Ion Torrent S5) of DNA. Sequences obtained were mapped to human reference genome GRCh37/hg19 and annotated using SOPHiA DDM platform.

Results: We considered the variants with ≥500X coverage and with a variant fraction (VF) ≥5%. Intronic, synonymous and 3'/5'UTR variants were filtered out. 18 samples were sequenced both on Illumina and Ion Torrent machines. A total of 262 variants (104 SNV and 158 INDELS) were found and of these 159 (61%) were detected by both the technologies. Positive controls validation: all known variants were properly confirmed by both the technologies. Illumina intra-run repeatability: 4 samples, 38 detected variants, 37 (97%) confirmed in both the replicates.

Ion Torrent S5 intra-run repeatability: 1 sample, 14 detected variants, 11 (79%) confirmed in both the replicates. Ion Torrent S5 inter-run repeatability: 4 samples, 61 detected variants, 46 (75%) confirmed in both the replicates. Two samples were sequenced on Ion Torrent S5 machine using both OT2/Enrichment System and Chef System to perform emulsion and enrichment steps. We found a total of 20 variants and 19 (95%) were detected in both the runs. Coverage analysis on 90% of the target regions: we obtained a median coverage of 2576X (range: 1171X-3478X) for the run1, 1806X (range: 1426X-1997X) for the run2, 1676X (range: 1456X-1951X) for the run 3 and 4252X (range: 3464X-4546X) for the run 4. Conclusion: MyS panel could be used in a diagnostic routine both on Illumina and Ion Torrent S5 machines. The low repeatability found for Ion Torrent S5 sequencer needs to be further investigated to better understand the factor affecting the results.

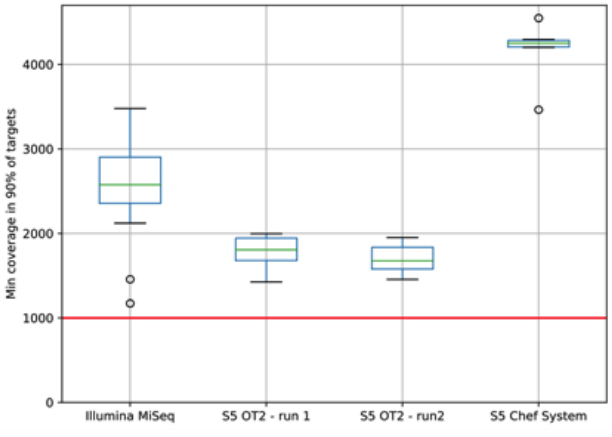

Figure 1. Minimum coverage on 90% of the sequenced target regions.

PO118

THE IMPROVED DIASORIN Q-LAMP ASSAY FOR THE ACCURATE AND ULTRA-FAST DETECTION OF COMMON AND RARE ISOFORMS OF THE BCR-ABL1 TRANSLOCATION

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Introduction: The molecular detection of BCR-ABL1 transcripts is mandatory to confirm the diagnosis of Philadelphia Positive Leukemias at onset. The most common molecular method for the detection of the BCR-ABL1 translocation is the nested RT-PCR but a faster and reliable assay based on the Q-LAMP technology developed by DiaSorin is entering in laboratory routine. This Q-LAMP assay is able to detect and discriminates in one hour the most common isoforms of BCR-ABL1 p190 (e1a2) and p210 (e13a2, e14a2). In this study, we evaluated the new improved Q-LAMP formulation designed to detect also less frequent isoforms of the BCR-ABL1 transcripts p190 and p210 (e1a3, e13a3, e14a3). In addition, clinical studies performed on the limited number of available patients have demonstrated that the assay is capable of detecting also the rare isoform p230 (e19a2, e19a3). Methods: The new Q-LAMP BCR-ABL assay consists in a multiplex assay for the differential detection of p190 and p210 transcripts (common and rare isoforms) and the simultaneous amplification of the GUSB endogenous RNA (internal control). The assay has been tested on a total of 166 clinical samples including 76 p210 positive (47 e13a2 and 19 e14a2, 8 e13a3 and 2 e14a3), 38 p190 positive (33 e1a2 and 5 e1a3) and 52 BCR-ABL1 negative samples. Additional 2 p230 (e19a2) rare isoforms were also included in this study. All samples were previously tested by nested RT-PCR (BIOMED 1 Protocol), considered in this study as the reference method. Results: The new enhanced BCR-ABL Q-LAMP assay showed 100% concordance with the nested RT-PCR, with a delayed amplification time for rare isoforms respect to the common ones. In particular the average amplification time of p210 common isoforms were 21,74 and 27,82 min compared to the p210 and p190 rare isoforms that showed average amplification times of 25,03 and 36,84 min, respectively. The 2 p230 (e19a2) rare isoforms were also tested resulting valid and due to the very long transcript they showed an average amplification time very high (50 and 48 min). All negative samples were confirmed by the exclusive amplification of the internal control. Moreover, although the clinical role of the e13a2 versus the e14a2 isoform has not been clearly established so far, we observed an interesting discrimination between these two isoforms in terms of amplification times (20,28 versus 25,37 min) maybe associated to the different length of the two transcripts, with low coefficients of variability (0,16 and 0,10 respectively). Conclusions: The enhanced BCR-ABL Q-LAMP assay well proved to be able to detect both common and uncommon isoforms of the BCR-ABL1 translocation. This improved performances, combined with the speed and the close tube format, allow laboratories in saving time and optimizing their workflow, representing a convenient and reliable solution for molecular diagnosis of Philadelphia Positive Leukemias.

Immunotherapy and cell therapy

PO119

GMP-ISOLATION AND CRYOPRESERVATION OF DONOR T REGULATORY CELLS FOR IMMUNOTHERAPY OF SEVERE REFRACTORY CHRONIC GVHD AFTER ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

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We are currently testing the role of multiple infusions of donor T regulatory cells (Tregs) in patients with refractory chronic Graft Versus Host Disease (cGVHD). So far, three patients have been treated with a dose of cryopreserved Tregs of 0,5x10^6 cells/Kg divided in three infusions. However, the effect of cryopreservation on Treg function is still poorly known.

Treg cells were purified with immunomagnetic selection in GMP conditions in the Cell Factory "Calori" then cryopreserved and shipped to Bologna. We have analysed the viability, the phenotype and the inhibitory capacity of purified donor Tregs before and after thawing. Furthermore we have analysed Treg numbers and phenotype in the peripheral blood (PB) of patients before and after (24 h later) infusion. All analyses have been performed by flow cytometry (FACS CANTO II, BD).

Following thawing we have observed a slight decrease of viability of donor Tregs (shown as% of cells 7-AAD-) : #1: 78,5% #2: 93 and #3: 75,5 (thawed Tregs) vs #1: 90%, #2: 95,9% ad #: 3 92,4% (fresh Tregs) (p=ns). The percentage of FoxP3 expression of thawed donor Tregs was stable, as was the percentage of CD15s expression. Instead, the percentage of CD62L expression was reduced in thawed donor Tregs (mean 52%) vs fresh donor Tregs (82%) ($p \leq 0.05$). Functionally, thawed donor Tregs inhibited the proliferation of autologous CD4+CD25- T cell (Teff) at different Treg:Teff ratios. The percentage of inhibition at the ratio 1:1(Treg:Teff) was 40%, 28% and 15% for each of the three patients. Most Treg infusion were associated with an increase of Treg numbers in the PB 24 hours later (6/9 infusions). Moreover, the number of Treg as well as the Treg/ Teff ratio was increased one month after the last infusion as compared to the baseline in one patient.

Use of donor cryopreserved GMP-isolated Tregs for immunotherapy of GVHD appears feasible. Cryopreserved Treg cells appear to maintain their phenotype and function. Moreover infusions of purifed Tregs may increase Treg numbers in the PB.

P0120

A SCREENING OF ANTINEOPLASTIC DRUGS FOR ACUTE MYELOID LEUKEMIA REVEALS THAT FLUDARABINE AND CYTARABINE HAVE WEAK CAPACITY AS INDUCERS OF IMMUNOGENIC CELL DEATH

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Introduction: Although antineoplastic drugs used as induction chemotherapy for patients with acute myeloid leukemia (AML) result in high rate of complete remission, disease relapse occurs in the majority of cases, unless stem cell transplant is not performed. Development of new therapies is, then, a major medical need. Among them, strategies harnessing the immune system against leukemia are under active investigation. It is known that the cancer cell death induced by some antineoplastics drugs, such as anthracyclines, is highly immunogenic and results in the maturation of dendritic cells (DCs) and in the efficient cross-priming of anti-tumor T cells. This process, named immunogenic cell death (ICD), is characterized by Calreticulin (CRT) and Heat Shock Proteins 70 and 90 (HSP70/90) translocation to cell membrane and release of HMGB1 protein from nucleus. In this study, we have *in vitro* compared ICD capacity of five among the most widely used drugs for AML.

Methods: HL-60 AML cell lines were treated with Daunorubicin (DNR), Cytarabine (Ara-C), Etoposide (VP-16), Fludarabine (Fluda) and Bortezomib. After apoptosis detection by flow cytometry, traslocation of CRT, HSPs70/90 and HMGB1 release from nucleus were evaluated using flow cytometry and immunofluorescence. Successively, the treated cells were used for loading into healthy donor-derived DCs which were used to induce T cell proliferation as evaluated by flow cytometry using CFSE.

Results: All five tested drugs induced a comparable levels of apoptosis. While Bortezomib and VP16 as well as DNR, which is a well-known ICD inducer, were capable to induce CRT and HSP70/90 traslocation and HMGB1 release from nucleus, Fluda and Ara-C have low, if any, capacity to induce ICD-related events. Importantly, only DNR, Bortezomib and VP16 treatment, but not Ara-C and Fluda, induced DC maturation, thus confirming that ICD-related events correlate with immunogenic changes in DCs. At the functional level, VP16 was comparable to DNR at eliciting T-cell proliferation via DCs, whereas Bortezomib, despite its capacity of inducing DC maturation via ICD, had low effect on T-cell proliferation. Again, Ara-C and Fluda had no effect on T-cell proliferation.

Conclusions: Our results indicate a novel and poorly-investigated feature of antineoplastic drugs, commonly used for the treatment of AML, which is based on their different immunogenic potential. Under this viewpoint, Fluda and Ara-C have weak, if any, capacity as inducers of immunogenic cell death, whereas VP16 and DNR are very effective. Further experiments, addressing the synergistic effects of these different drugs, may help to combine their conventional cytotoxic activity with their immunogenic capacity, thus providing the platform for a novel approach to anti-leukemia chemotherapy.

P0121

MULTIDIMENSIONAL GERIATRIC ASSESSMENT FOR ELDERLY PATIENTS (> 60 YEARS) SUBMITTED TO ALLOGENEIC STEM CELL TRANSPLANTATION. A SINGLE CENTER ANALYSIS ON 69 PATIENTS

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Background: Patients (pts) selection remains a big deal in allogeneic stem cell transplantation (allo-SCT), especially in the subset of elderly pts. In this category, the availability of scores predictive for outcome is strongly warranted.

Aims: To evaluate the impact of sole comorbidity scores (Sorror HCI and Charlson Comorbidity index CCI) and combined comorbidity and functional scores (Balducci and FIL scores) on allo-SCT outcome in elderly patients.

Patients and Methods: Clinical data of 69 pts older than 60y submitted to allo-SCT for hematological neoplasms between 2006 and 2017 at Unit of Bone Marrow Transplantation of Spedali Civili di Brescia were retrospectively collected. All pts received HCI and CCI and 63 (91%) Balducci and FIL geriatric multidimensional tests (Balducci L & Extermann M The Oncologist 2000; Tucci A et al. Leuk&Lymph 2015) before transplantation.

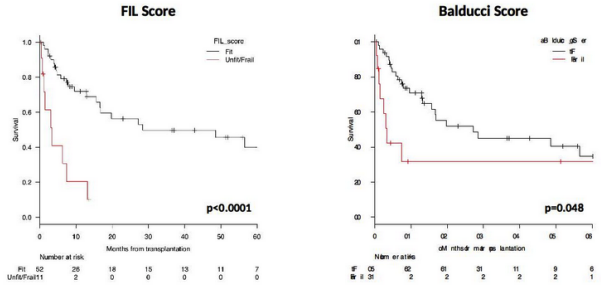

Figure 1.

Results: Clinical characteristics of the cohort were the following: median age 63 years (range, 60-70), male gender 45%, acute leukemia 48 (70%), CR status 44 (64%), female donor to male recipient 11 (16%); sibling donor 29 (42%), matched unrelated 29 (42%), alternative donor 11 (15%); peripheral blood as stem cell source 50 (72%), bone marrow 18 (26%), cord 1 (2%); Myeloablative conditioning regimen 5 (7%), CD34+ x10^6/Kg cells 4,8 (0,7-6,7), CD3+ $x10^7$/Kg cells 13,2 (0,01-47,8), median follow-up 13 months (1-138). The 2y overall survival (OS), transplant related mortality (TRM) and cumulative incidence of relapse (RI) were 45%, 12% and 49%, respectively. A total of 39 (57%) pts had died at last follow-up. Causes of deaths were: transplant-related 23% (aGVHD 1, cGVHD 1, infection 12, toxicity 2), disease relapse 32%, other unrelated 1%. According to Sorror HCI, 26 pts (38%) had a score 0, 11 (16%) 1, 5 (7%) 2, 27 (39%) pts had a score higher than 2. CCI distribution was: 0 (23%), 1 (35%), 2 (20%), >2 (22%). Both Sorror HCI (HR 1.01, CI95% 0.85-1.20, p=0.90) and CCI (HR 0.96, CI95% 0.74-1.25, p=0.77) failed in predicting allo-SCT outcome in our cohort FIL score classified pts as fit in 52 (75%) and unfit/frail in 11 cases (25%), Balducci as frail in 13 (21%) and unfrail in 50 cases (79%), respectively. No differences were detected between fit and unfit patients in terms of alloSCT features. Multidimensional scores were able to highly predict survival. In fact, fit patients according to FIL score were projected to a OS of 56% compared to 10% at 2y of unfit/frail pts. Similarly, unfrail pts according to Balducci score had an OS of 52% compared to

32% at 2y (Figure 1). Interestingly, TRM did not significantly differ according the two scores; on the contrary, a significant higher relapse incidence was recorded in unfit/frail pts according FIL score (RI 71% vs 41% at 2y, p=0.004).

Conclusions: In our experience, a geriatric multidimensional approach seems to be more accurate compared to the sole comorbidity assessment in predicting elderly pts outcome after allo-SCT.

PO122

CHARACTERIZATION OF HUMAN BONE MARROW NESTIN-POSITIVE VASCULAR NETWORK

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Introduction: Bone marrow is a complex, sponge-like tissue characterized by stem cells with haematopoietic activity. Haematopoietic stem cells (HSCs) reside in special microenvironment known as niches that regulate HSC biological behaviour, influencing self-renewal and differentiation. Two different niches have been described; one in close proximity of endosteum and the other located near the vessels. However, today the existence of two different niches is questioned because of the high vascularisation of the endosteal region that probably closely connects them in a continuum. Recently, nestin expression has been applied to distinguish arteriolar vessels from sinusoids, and these two distinct types of microvessels have been described in correlation to different HSCs subpopulation, in mice. Based on the nestin expression, the bone marrow microvessels are newly classified in arterial nestin positive vessels and nestin negative sinusoids.

Methods: In this work, nestin expression has been evaluated in paraffin-embedded human bone marrow biopsies from patients with non-Hodgkin lymphoma. Histological analysis was performed applying Immunohistochemical techniques, using confocal microscope and image analysis.

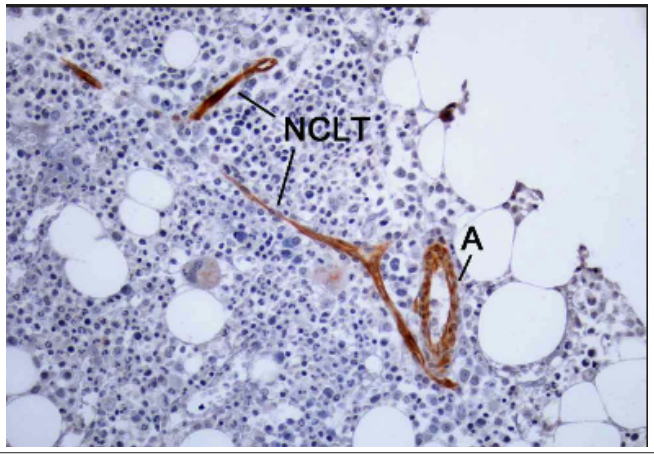

Figure 1.

Results: Nestin expression has been found in human bone marrow arterial vessels, while sinusoids resulted negative. Bone marrow arteries (A) and endosteal arterioles (EA), previously described in mice, has been confirmed also in human, maintaining similar classification parameters, based on the inner caliber. In addition to these, here we also described a narrower capillary-like structure that we named nestin-positive capillary-like tubes (NCLT), and single or clustered cells indicated as nestin-positive cells (NC). The complexity of all these structures has been investigated analyzing CD146-positive accessory cells, founding an association between complexity of second layer and A e EA; while NCLT and NC resulted not associated with CD146-positive cells. In parallel, the involvement of newer nestin-positive structures in the haematopoietic niche has been evaluated by quantification and estimating the distance from surrounding CD34-positive HSCs. Results showing similar distribution around NCLT and NC to that reported for EA.

Conclusions: These findings suggest that the classification proposed for mouse BM vessels can be transposed to human BM. Additionally, new previously not described NCLT and NC could be hypothesized to be involved in vascular niche regulation, similarly to A and EA. This finding is of particular interest taking into consideration that the HSC interaction with A or EA, seems to be mediated by the pericytic population, absent around NCLT and NC. This could also suggest a direct interaction between HSC and NCLT endothelial cells or NC.

PO123

POMALIDOMIDE-RELATED NEUTROPENIA IN REFRACTORY MYELOMA PATIENTS IS ASSOCIATED TO IMPROVED NEUTROPHIL FUNCTION

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Backgound: Pomalidomide is a novel immune-modulatory drug approved for relapsed/refractory multiple myeloma patients (RRMM), with drug-related neutropenia as major limitation to treatment. Our group previously showed that neutrophils are dysfunctional in MM, with an aberrant immune-phenotype.

Aim: to investigate immune-phenotype and absolute count of neutrophils in RRMM undergoing salvage treatment with pomalidomide cointaning regimens.

Patients and methods: Herein, we describe a retrospective analysis of 51 consecutive RRMM patients treated with pomalidomide and dexamethasone, from November 2014 through January 2016, receiving subcutaneous G-CSF as part of prophylaxys regimen when leukocytes count was $\leq 2.5 \cdot 10^9$/L and neutrophils count was $\leq 1.5 \cdot 10^9$/L. PomaD regimen consisted in pomalidomide given 4 mg daily per os on days 1-21 of each 28-day cycle and dexamethasone 40 mg weekly (for <75 years patients) and 20 mg weekly (for ≥ 75 years patients) until progression.

Results: 33/51 patients (median age 68 years, range 42-78), heavily pre-treated (median number of previous therapies 4, range 2-7) required supportive care with G-CSF administration. 29 patients had pre-existing severe anemia, 5 thrombocytopenia, 15 neutropenia, related to bone marrow infiltration and severe toxocity of previous treatments. A median number of 8 (range 1-21) PomaD cycles were given. Overall treatment mean duration was 7.7 months. About one third of the patients responded within first two cycles. After a median follow-up of 12 months, median PFS and OS for patients were 6.7 and 9.9 months, respectively. Hematological adverse events occurred only in 10% of cycles, including anemia (5%), thrombocytopenia (3.5%) and neutropenia (9%). The reduced frequency of neutropenia was attributed to the use of our prophylaxis policy. At baseline, RRMM-neutrophils showed grains and nuclei morphology suggestive of a defective maturation process. In addition, they showed reduced phagocytic activity and oxidative burst together with an aberrant expression of neutrophil activation markers CD64. After 4 cycles of PomaD CD64 was further increased, but phagocytic activity and oxidative burst recovered completely, improving up to levels comparable to those of healthy subjects, in both groups of patients receiving or not G-CSF. In patients who did not receive G-CSF (N=15/18), relative decreases in the neutrophil count were inversely associated with relative increases in the intensity of CD64 (r=- 0.32; p=0.03) and reduction of CD16 (r= 0.52; p=0.02) median intensity of fluorescence detected by flow cytometry.

Conclusions: Our data suggest that in MM patients treated with the combination of pomalidomide and dexamethasone, intensification of G-CSF prophylaxis can reduce frequency of serious adverse events and enable full dosage of pomalidomide. After four cycles of PomaD, neutrophil function improved with recovery of phagocytic activity and oxidative burst.

P0124

PEGFILGRASTIM VERSUS FILGRASTIM IN THE MANAGEMENT OF THERAPY RELATED NEUTROPENIA IN RELAPSED AND REFRACTORY MULTIPLE MYELOMA IN TREATMENT WITH POMALIDOMIDE-DEXAMETHASONE

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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. The objective of this study was to compare the efficacy and safety of pegfilgrastim in patients affected by heavily pretreated MM, treated with pomalidomide-dexamethasone, 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 29 patients (17 male and 12 female) median age at diagnosis 69 years (r. 52-84), and median age at start of treatment 76 years (r.56-89) treated with several lines of treatments (median 7, r. 2-11), every refractory to all the drugs previously received, received Pomalidomide-Dexamethasone (Pomalidomide 4 mg for 21 days, Dexamethasone 40 mg days 1,8,15,22, pegfilgrastim day +8) every 28 days, until progression. Since first course, received in domestic setting, with a very good compliance, patients performed blood counts once weekly and received, from day +8 to day +19 ("day + 1" when the protocol starts), prophylactic oral chinolonic antibiotics and anti-fungal drugs. During neutropenia after first cycle, Filgrastim (5 μgr/kg/day for 3 days) was given if neutrophils count was <1500 $x10^9$ cells/L. Median number of filgrastim administrations was 4.7 (r. 3-6); nadir neutropenia was registered after a median of 10.3 days (r. 7-14); median of nadir neutrophil count was $1.13x10^9$ cells/L (range 0.3 – $1.5x10^9$ cells/L), with maximum duration of 14 days. From the second course, all patients switched to prophylaxis with pegfilgrastim (6 mg), injected subcutaneously with a single administration on day +3 independently from the neutrophil count at that time. Primary endpoint of this study was the duration of neutropenia (neutrophil count < 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 1.37 (range 0.9-$2.1x10^9$ cells/L); only 4 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, (6/29 patients, 20.6%)

In conclusions, in patients affected by heavily pretreated MM treated with pomalidomide-dexamethasone, pegfilgrastim seems to reduce the incidence of severe neutropenia and infections and may increase the possibility to maintain the scheduled time of treatment.