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Molecular Genetics of Myeloid Malignancies Drives Modern Treatment Options

Salmoiraghi Silvia

Registration Number 863325

Tutor: Dott. Martino Introna

Coordinator: Prof. Andrea Biondi

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CHAPTER 1

INTRODUCTION

Cancer is a leading cause of death worldwide and particularly in developed countries, accounting for nearly 10 million deaths in 2020, or nearly one in six deaths. Cancerogenesis is a multi-step disease arising from the transformation of a normal cell into tumor cells characterized by an abnormal and unregulated proliferation. During tumorigenesis, alterations/mutations in genes or gene expression lead to a dysregulation of cell cycle checkpoints which cause a cell to grow in an uncontrolled manner. The generalized loss of growth control exhibited by tumor cells is the result of accumulated abnormalities in multiple cell regulatory systems and is reflected in several aspects of cell behavior that distinguish cancer cells from their normal counterparts. (1) The great efforts in oncology research and the huge technological advances have greatly increased our understanding of the molecular basis of tumorigenesis, tumor progression and responses to different available treatments. The great challenge characterizing oncology is translating the results obtained from basic biological studies into advances in patients' care. Over the last 20 years, the increasing information on cancer genetics and biochemistry have led to the identification of several different tumor biomarkers. Particularly, prognostic markers aim to evaluate the patient's overall outcome, such as the probability of cancer recurrence after standard treatment. The presence or absence of a prognostic marker can be useful for the selection of patients for treatment but does not directly predict the response to a treatment. Predictive markers aim to objectively evaluate the likelihood of benefit from a specific clinical intervention, or the differential outcomes of two or more interventions, including toxicity. The evaluation of biomarkers has an increasing importance in the decision making for the management of the oncologic patient and, for this reason,

biomarkers are frequently included in risk scoring systems developed to classify patients during the disease course.(2)

This work is focused on myeloid neoplasms and the aim of the studies conducted during my PhD internship was to better define the role of genetic mutations to predict the clinical course of the disease in order to guide the choice of the best clinical strategy based on cancers' biologic characteristics as well as on the clinic.

1. MYELOID NEOPLASMS: DEFINITION AND CLASSIFICATION

Myeloid malignancies are a heterogeneous group of clonal diseases of the hematopoietic stem cells originating, in particular, from myeloid precursors. (3) These malignancies result from genetic and epigenetic alterations that perturb key cellular processes such as self-renewal, proliferation, differentiation and apoptosis. (4) Myeloid neoplasms include myeloproliferative neoplasms (chronic myeloid leukemia, polycythemia vera, essential thrombocythemia, primary myelofibrosis, and mastocytosis), myelodysplastic syndromes, and acute myeloid leukemia. Myeloproliferative neoplasms and myelodysplastic syndromes can transform to secondary acute myeloid leukemia. (5) For this reason, acute myeloid leukemia (AML), have long been classified as malignant while other myeloid disorders have been considered non-malignant or preleukemia blood disorders which may progress to a malignant condition over time. More recently, scientific advances in understanding these diseases indicates

that these “pre-leukemia” blood disorders are actually forms of slow-growing blood cancers. (6), (7)

Basing on clinical features combined with morphology, cytochemistry, immunophenotype and genetic characteristics, the World Health Organization (WHO) categorizes myeloid malignancies into five primary types: AML, myelodysplastic syndromes (MDS), myeloproliferative neoplasms (MPN), myelodysplastic and myeloproliferative (MDS/MPN) neoplasms and mastocytosis.

In this work, we were focused particularly on AML, primary or secondary to a previous MPN or MDS and on Myelofibrosis (MF), primary or evolved from Polycytemia Vera (PV) or Essential Thrombocythemia (ET).

Acute Myeloid Leukemia (AML)

AML is a hematological disease that originates from the hematopoietic stem cell. The myeloid precursor with the capacity to self-renew undergoes a serial alteration of the mechanisms that regulate cell proliferation and differentiation leading to a neoplastic transformation. This results in an accumulation of proliferating blasts in the bone marrow and subsequently in the peripheral blood. Approximately 18,300 cases of AML are diagnosed each year in Europe and the annual incidence is 2-4 cases in 100,000. (Parkin et al., 2002) AML is a disease that can arise at any age, although the highest incidence is reported in the decade between 50 and 60 years.(9) Several agents potentially associated with the onset of LAM have been identified, such as smoke, benzene and its derivatives and ionizing radiation. In

addition, exposure to alkylating drugs, used in some chemotherapy programs, increases the risk of developing AML. In addition, some genetic diseases (Fanconi anemia, Bloom syndrome, neurofibromatosis) and blood disorders (chronic myeloproliferative disorders and myelodysplastic syndrome) can increase the risk of developing this disease. In young and fit individuals affected by AML, the standard treatment consists of intensive chemotherapy combining cytarabine and an anthracycline, with or without consolidative allogeneic hematopoietic stem cell transplant (aHSCT). Recently an improved understanding of the biology and molecular heterogeneity of AML has led to the development of novel low-intensity and molecularly targeted therapies for patients who cannot be considered eligible for standard approaches.(10)

1.1.1 **AML Classification**

Classification of AML has been recently updated and the major change is represented by the separation of AML defining by genetic abnormalities from AML defined by differentiation, as a consequence of the increasing understanding of this disease associated with the progress of molecular genetics.(11). Another key change introduced by this recent revision is the elimination of the 20% of blasts as a requirement for diagnosis of AML defined by the presence of genetic abnormalities, excepted for AML with *BCR/ABL1* fusion and AML with *CEBPA* mutation. However, consequently to the blast cutoff removal, the integration of information regarding morphologic and immunophenotipical aspects with genetics is crucial to ensure that genetic abnormalities are driving the disease pathology.

1.1.1.1 WHO 2022 Classification for AML defining by genetic abnormalities

Cytogenetic analysis plays an important role in the diagnosis of AML showing most of the numerical and structural chromosomal abnormalities that are present in 50-70% of adult AML cases. (12) Chromosomal rearrangements can give rise to fusion transcripts that can also be identified by molecular analysis. The main fusion genes derived from balanced translocations are: *RUNX1-ETO* [t (8; 21)], *CBFβ-MYH11* [inv (16)], and *PML-RAR* [t (15; 17)] in Acute Promyelocytic Leukemia. Furthermore, in AML, several alterations in genes involved in different biological functions have been described. The identification of these mutations plays a fundamental role in the classification of AML and in predicting the clinical course of the disease. (11)

Basing on the genetic characteristics at diagnosis, AML definition by genetic abnormalities is reported in Table 1

Acute myeloid leukemia with defining genetic abnormalities
Acute promyelocytic leukemia with PML/RARA fusion
Acute myeloid leukemia with RUNX1/RUNX1T1 fusion
Acute myeloid leukemia with CBFB/MYH11 fusion
Acute myeloid leukemia with DEK/NUP214 fusion
Acute myeloid leukemia with RBM15/MRTFA fusion
Acute myeloid leukemia with BCR/ABL1 fusion
Acute myeloid leukemia with KMT2A rearrangement
Acute myeloid leukemia with MECOM rearrangement
Acute myeloid leukemia with NUP98 rearrangement
Acute myeloid leukemia with NPM1 mutation
Acute myeloid leukemia with CEBPA mutation
Acute myeloid leukemia, myelodysplasia-related
Acute myeloid leukemia with other defined genetic alterations

Table 1: AML Classification by genetic abnormalities by WHO 2022

AML with *BCR/ABL1* and AML with *CEBPA* mutation are the only types with a defined genetic abnormality in which the definition of at least 20% blasts is mandatory for diagnosis. The blast cut-off requirement is necessary for the former to avoid overlap with CML. Conversely, data supporting any change in the blast cut-off definition for AML with mutated *CEBPA* diagnosis are still not sufficient. Moreover, the definition of AML with *CEBPA* mutation was recently updated, as a consequence of the newly emerged data regarding mutation involving basic leucine zipper (bZIP) region of this gene. In fact, the AML characterized by a single mutation in the bZIP domain were demonstrated to be associated to a favorable outcome in pediatric cohort and adults up to 70 years. (13,14)

The myelodysplasia-related AML (AML-MR) category has been recently well defined conjugating morphology with biologic data. This specific type of AML is defined as a neoplasm characterized by at least 20% blasts expressing a myeloid immunophenotype and carrying specific cytogenetic and molecular lesions typically associated with MDS. This leukemia can arise *de novo* or as a progression of a previously MDS or myeloproliferative disorders. Cytogenetic and molecular abnormalities associated with MDS are summarized in Table 2.

In addition, a specific subtype of AML was introduced and defined as *new and/or uncommon AML subtypes*. This generic definition gives the possibility to include AML characterized by genetic/molecular lesions still not well described in AML classification. This is an “open” category in which any uncommon mutation can be included to categorize AML subtype.

Hypothetically, a new definition of a AML subtype may derive from this subgroup in the future.(11)

Cytogenetic and molecular abnormalities defining Myelodysplasia-related AML
Complex karyotype (≥3 abnormalities)
5q deletion or loss of 5q due to unbalanced translocation
Monosomy 7, 7q deletion, or loss of 7q due to unbalanced translocation
11q deletion
12p deletion or loss of 12p due to unbalanced translocation
Monosomy 13 or 13q deletion
17p deletion or loss of 17p due to unbalanced translocation
Isochromosome 17q
idic(X)(q13)
Somatic mutations involving *ASXL1, BCOR, EZH2, SF3B1, SRSF2, STAG2, U2AF1, ZRSF2*

Table 2: Genetics abnormalities defining myeloid leukemia myelodysplasia-related

1.1.1.2 WHO 2022 Classification for AML defining by differentiation

Although the number of cases of AML that are not characterized by a genetic abnormality will decrease more and more with the increasing knowledge concerning AML related genetics, some cases of AML that lack defining genetic abnormalities are still present and need a classification based on differentiation. This classification takes into account different markers (defined by immunophenotyping or cytochemistry) and criteria (clinical and

morphological) that define the AML differentiation, as indicated by the table below (Table 3).

Acute myeloid leukemia, defined by differentiation	Diagnostic Criteria
Acute myeloid leukemia with minimal differentiation	Blasts are negative (<3%) for MPO and SBB by cytochemistry Expression of two or more myeloid associated antigens, such as CD13, CD33 and CD117
Acute myeloid leukemia without maturation	≥3% blasts positive for MPO (by immunophenotyping or cytochemistry) or SBB and negative for NSE by cytochemistry Maturing cells of the granulocytic lineage constitute Expression of two or more myeloid-associated antigens, such as MPO, CD13, CD33, and CD117
Acute myeloid leukemia with maturation	≥3% blasts positive for MPO (by immunophenotyping or cytochemistry) or SBB by cytochemistry Maturing cells of the granulocytic lineage constitute ≥10% of the nucleated bone marrow cells Monocyte lineage cells constitute < 20% of bone marrow cells Expression of two or more myeloid-associated antigens, such as MPO, CD13, CD33, and CD117
Acute basophilic leukemia	Blasts & immature/mature basophils with metachromasia on toluidine blue staining Blasts are negative for cytochemical MPO, SBB, and NSE No expression of strong CD117 equivalent (to exclude mast cell leukemia)
Acute myelomonocytic leukemia	≥20% monocytes and their precursors ≥20% maturing granulocytic cells ≥3% of blasts positive for MPO (by immunophenotyping or cytochemistry)

Acute monocytic leukemia	≥80% monocytes and/or their precursors (monoblasts and/or promonocytes) <20% maturing granulocytic cells Blasts and promonocytes expressing at least two monocytic markers including CD11c, CD14, CD36 and CD64, or NSE positivity on cytochemistry
Acute erythroid leukemia	≥30% immature erythroid cells (proerythroblasts) Bone marrow with erythroid predominance, usually ≥80% of cellularity
Acute megakaryoblastic leukemia	Blasts express at least one or more of the platelet glycoproteins: CD41 (glycoprotein llb), CD61 (glycoprotein IIIa), or CD42b (glycoprotein lb)b

Table 3: Classification of AML by differentiation by WHO 2022.

Shared diagnostic criteria include: - ≥20% blasts in bone marrow and/or blood (except for acute erythroid leukemia). - Criteria for AML types with defined genetic alterations are not met. - Criteria for mixed-phenotype acute leukemia are not met (relevant for AML with minimal differentiation). - Not fulfilling diagnostic criteria for myeloid neoplasm post cytotoxic therapy. - No prior history of myeloproliferative neoplasm. BM bone marrow, MPO myeloperoxidase, NSE nonspecific esterase, PB peripheral blood, SBB Sudan Black B

1.1.1.3 WHO 2022 Classification for Secondary Myeloid Neoplasms

Differently from the previous classification (WHO 2016), in this category neoplasms are included myeloid neoplasms arising secondary to exposure to cytotoxic therapy or to a genetically determined predisposition. As already mentioned, in fact, the AML transformed from a previous myeloproliferative disorder (MPN, MDS or MDS/MPN) are classified as MPN or peculiar AML (myelodysplasia-related AML). (11)

Myeloid neoplasms post cytotoxic therapy category includes AML, MDS and MDS/MPN arising in patients previously exposed to cytotoxic drugs for independent disease conditions. Recent findings allow to understand that a significant number of therapy related AML develop through clonal selection of pre-existing hematopoietic cell clone secondary to a selection pressure of cytotoxic drugs in an impaired morrow environment (15) or to mutational changes induced by cytotoxic exposure. Therapy related AML are frequently associated to an alteration in *TP53* gene, that could be bi-allelic, thus involving 2 alleles. This double-hit aberration are associated with a worse clinical outcome.

1.1.1.4 WHO 2022 Classification for AML with myeloid predisposition

Myeloid neoplasms associated with germline predisposition include AML, MDS, MPN, and MDS/MPN that are diagnosed in individuals with genetic conditions associated with increased risk of develop a myeloid malignancy. These AML are now classified combining the myeloid disease phenotype with the predisposing germline genotype. The categories are the following:

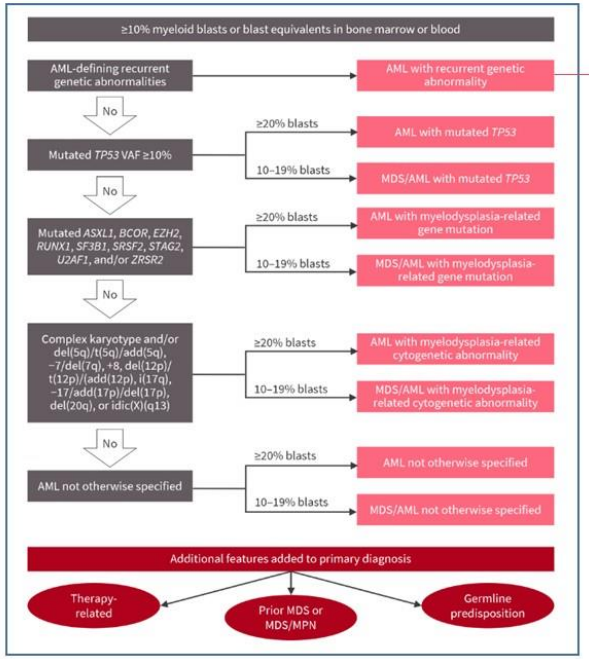
- Myeloid neoplasms with germline predisposition without a preexisting platelet disorder or organ dysfunction
- Myeloid neoplasms with germline predisposition and pre-existing platelet disorder
- Myeloid neoplasms with germline predisposition and potential organ dysfunction

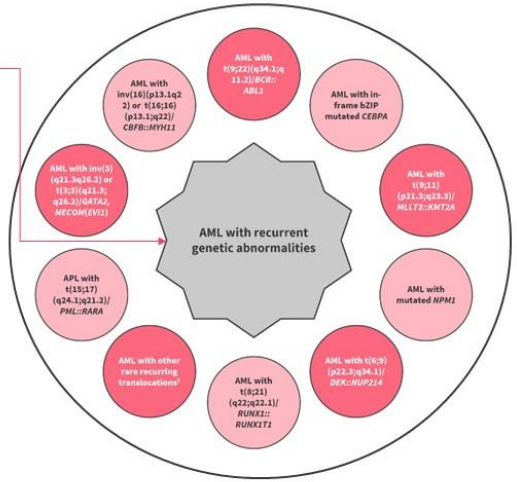
Each subtype is associated with peculiar clinical manifestations and also to peculiar genetic alterations involving different genes. (11)

1.1.1.5 AML Classification according to International Consensus Classification (ICC)

In parallel with the latest classification of AML by the WHO, a new version of recommendations by European Leukemia Net (ELN) for the management of AML in general clinical practice and for clinical trials was published. These recommendations were released as a result of the recent advances in the understanding of AML genetic landscape, in the development of assays for genetic characterization and MRD detection as well as in the development of novel drugs with anti-leukemic effect. (16)

ICC changed the previous definition based on 20% of blasts to define an AML, when recurrent genetic lesions is identified. Indeed, while the blast threshold remains at 20% for the majority of AML subtypes, the presence of one of the recurrent genetic lesions, are now considered to define an AML if blasts are ≥10% in bone marrow or peripheral blood. Particularly, the algorithm reported in Figure 1 shows that the presence of specific genetic abnormalities defining myelodysplasia-related phenotype (also reported in Table 2) allows to classify the myeloid malignancy as AML if there are 20% blasts or as AML/MDS if the blast count is 10–19% (Figure 1) This new category includes cases previously classified as AML with myelodysplasia-related changes (AML-MRC); the AML-MRC subgroup has been removed as genetic characteristics are now considered more relevant than clinical ones.





*Other rare recurring translocations include AML with t(1;3)(p36.3;q21.3)/PRDM16::RPN1, AML (megakaryoblastic) with t(1;22)(p13.3;q13.1)/RBM15::MRTF1, AML with t(3;5)(q25.3;q35.1)/NPM1::MLF1, AML with t(5;11)(q35.2;p15.4)/NUP98::NSD1, AML with t(7;12)(q36.3;p13.2)/ETV6::MNX1, AML with t(8;16)(p11.2;p13.3)/KAT6A::CREBBP, AML with t(10;11)(p12.3;q14.2)/PICALM::MLLT10, AML with t(11;12)(p15.4;p13.3)/NUP98::KMD5A, AML with NUP98 and other partners. AML with t(16;21)(p11.2;q22.2)/FUS::ERG, AML with t(16;21)(q24.3;q22.1)/RUNX1::CBFA2T3, and AML with inv(16)(p13.3q24.3)/CBFA2T3::GLIS2

Figure 1: Hierarchical classification of AML by ICC, adapted from Dohner 2022*

This new classification allows patients with low-blast count myeloid leukemia to access treatment approaches for both MDS and AML.

1.1.2 **AML diagnostic procedure**

ELN recommendations also suggest the diagnostic work up for AML is a specific multi-step procedure that consider different aspects of the disease and require different diagnostic approaches, listed below.(16)

Expression of cell-surface and cytoplasmic markers	
Diagnosis of AML	
Precursors	CD34, CD117, HLA-DR
Myeloid markers	CD33, CD13, cytoplasmic MPO
Myeloid maturation marker	CD11b, CD15, CD64, CD65
Monocytic markers	CD14, CD36, CD64, CD4, CD38, CD11c
Megakaryocytic markers	CD41 (glycoprotein IIb/IIIa), CD61 (glycoprotein IIIa),CD36
Erythroid markers	CD235a (glycophorin A), CD36, CD71
Diagnosis of MPLA	
Myeloid lineage	MPO (flow cytometry, immunohistochemistry, or cytochemistry) or monocytic differentiation (at least 2 of the following: nonspecific esterase cytochemistry, CD11c, CD14, CD64, lysozyme) or at least 2 myeloid markers
T-lineage	Strong cytoplasmic CD3 (with antibodies to CD3 e chain) or surface CD3
B-lineage	Strong CD19 with at least 1 of the following strongly expressed: cytoplasmic CD79a, cCD22, or CD10 or weak CD19 with at least 2 of the following strongly expressed: CD79a, cCD22, or CD10.

Table 4: Expression of cell surface and cytoplasmatic markers for the diagnosis of AML and MPAL

Morphology: A morphologic evaluation of at least 200 leukocytes on blood smears and 500 nucleated cells on marrow smears should be performed at AML diagnosis.

Immunophenotyping: The immunophenotypic analysis is carried on by applying flow cytometry performed using specific antibodies that recognize markers on cell surface or in cytoplasm. The use of specific antibodies allows to identify the different cell populations and therefore to better characterize the AML subtypes. Table 4 summarizes the expression of cell-surface and cytoplasmic markers for the diagnosis of AML and Mixed Phenotype Acute Leukemia (MPAL).

Cytogenetic and molecular biology: Cytogenetic analyzes play an important role in the diagnosis of AML. They can reveal most of the numerical and structural chromosomal abnormalities that are present in 50-70% of AML cases in adults. (17) Identification of cytogenetic abnormalities allows patients classification of AML as recently described by the new WHO classification. Moreover, cytogenetics is also essential to classify AML into different risk classes on the basis of the associated prognosis. When conventional cytogenetic analysis fails, fluorescence in-situ hybridization can represent a valid alternative to detect specific abnormalities like *PML/RARA*, *BCR/ABL1*, *RUNX1/RUNX1T1*, *CBFB/MYH11*, *KMT2A (MLL)*, and *MECOM (EVI1)* gene fusions, or myelodysplasia-related chromosome abnormalities. Chromosomal rearrangements can produce fusion transcripts that can also be identified by molecular analysis, particularly RT-

PCR. The main fusion genes derived from balanced translocations are: *RUNX1-ETO* [t (8; 21)], *CBFβ-MYH11* [inv (16)], and *PML-RAR* [t (15; 17)] in APL. Most frequent rearrangements characterizing AML and used for its classification are reported in Table 1.

Furthermore, several molecular alterations in genes involved in different biological functions have been described in AML. (18) The screening of the following genes for mutation detection is required for establishing the diagnosis and to identify actionable therapeutic targets: *FLT3*, *IDH1*, *IDH2* and *NPM1* (in 3-5 days from the diagnosis to start the adequate therapy scheme) and *CEBPA*, *DDX41*, *TP53*, *ASXL1*, *BCOR*, EZH2, *RUNX1*, *SF3B1*, *SRSF2*, *STAG2*, *U2AF1* and *ZRSR2* (within the end of the first induction cycle). The identification of these mutations plays a fundamental role for classification, for prediction of the course of the disease and therefore in the choice of the therapeutic strategy. The detection of alterations involving different genes requires the application of NGS approaches allowing the simultaneous study of several extended genomic regions. Different gene panels were designed and became commercially available for AML characterization. Some of them give also the opportunity to analyze molecular mutations and rearrangements by an all-in-one solution. The detection of Internal Tandem Duplication of *FLT3* (FLT3-ITD) by NGS approaches could fail for technical limitations. It is, therefore, necessary to apply an alternative method suitable for the identification of long insertion (>50 base pairs), for example capillary electrophoresis. Furthermore, differently from what previously reported (19), only in-frame mutations affecting the basic leucine zipper (bZIP) region of *CEBPA*, irrespective

whether they occur as monoallelic or biallelic mutations, have been associated with favorable outcome. Moreover, if a AML with germline predisposition is suspected, the application of a gene panel allowing the identification of common predisposition alleles should be applied. These panels should investigate at least these gene regions as target of sequencing; *ANKRD26, BCORL1, BRAF, CBL, CSF3R, DNMT3A, ETV6, GATA2, JAK2, KIT, KRAS, NRAS, NF1, PHF6, PPM1D, PTPN11, RAD21, SETBP1, TET2, WT1.* However, a special attention must be paid in evaluating sequencing results in suspected hereditary cases for 2 reasons: hematopoietic tissues can undergo somatic reversion leading to false-negative results and predisposing alleles characterized by copy number variants are often undetectable by conventional NGS panel. Finally, in the diagnostic work-up of AML, it is also necessary a quantification of *NPM1* mutated allele as well as a quantification of fusion transcripts characterizing core binding factor AML in order to set a baseline to use for MRD assessment at different time-points.

1.1.3 **AML risk classification by European Leukemia Net (ELN 2022)**

As known and already considered in the previous versions of ELN risk classification, genetic and molecular abnormalities are powerful prognostic markers. (20) (18) Particularly, conventional cytogenetic and mutational status of *NPM1*, *FLT3* and *CEBPA* have been considered since 2010 in the routine practice as suggested by 2nd version of ELN recommendations. (21) In the 3rd version of ELN published in 2017, also the alterations involving *ASXL1*, *RUNX1* and *TP53* were taken into consideration as an adverse marker

of clinical outcome. (22) The risk classification based on genetics features have been recently implemented at diagnosis latest version of ELN considering data on AML molecular landscape. (23–25) Table 5 describes genetic abnormalities defining the risk category ELN recommendation 2022(11)

Risk Category	Genetic Abnormalities
Favorable	t(8;21)(q22;q22.1)/RUNX1::RUNX1T1 inv(16)(p13.1q22) or t(16;16)(p13.1;q22)/CBFB::MYH11 Mutated NPM1 without FLT3-ITD bZIP in-frame mutated CEBPA
Intermediate	Mutated NPM1 with FLT3-ITD Wild-type NPM1 with FLT3-ITD t(9;11)(p21.3;q23.3)/MLLT3::KMT2A Cytogenetic and/or molecular abnormalities not classified as favorable or adverse
Adverse	(6;9)(p23;q34.1)/DEK::NUP214 t(v;11q23.3)/KMT2A-rearrangedg t(9;22)(q34.1;q11.2)/BCR::ABL1 t(8;16)(p11;p13)/KAT6A::CREBBP inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2)/GATA2, MECOM(EVI1) t(3q26.2;v)/MECOM(EVI1)-rearranged -5 or del(5q); -7; -17/abn(17p) Complex karyotype,h monosomal karyotype Mutated ASXL1, BCOR, EZH2, RUNX1, SF3B1, SRSF2, STAG2, U2AF1, or ZRSR2

Table 5: ELN 2022 risk classification by genetics at baseline

In addition to these genetic characteristics investigated on the diagnostic sample, response to initial therapy and assessment of early MRD need to be considered as a prognostic factor. Various studies and a systematic meta-

analysis have shown the prognostic value of MRD for relapse and overall survival (OS). (26, 27).

The evaluation of MRD in AML is oriented to:

1) Provide a quantitative method to investigate a molecular remission status
2) Estimate a post remission relapse risk to guide the therapeutic choice for consolidation therapy
3) Identify impending relapse that can be treated with an early intervention.

Different methodologies can be applied for MRD assessment. Multi-parameter flow cytometry and quantitative PCR for detection of *NPM1* mutated allele as well as chimeric transcripts are the most robust. In the last few years, also alternative approaches for MRD assessment were evaluated and resulted promising. Particularly, digital PCR can be a valid option thanks its potentiality to detect the majority of the somatic mutation, with limited costs. Moreover, also NGS approaches are under investigation (ref), even if, at present, they resulted less sensitive than the others previously described, more expensive and technically challenging.(28)

1.1.4 **AML Therapy**

The goal of AML treatment is to control and, when possible, to eradicate the disease. Ideally, AML therapy implies an initial treatment that induces the Complete Remission (CR) achievement, followed by a consolidation and/or a maintenance. The choice of therapy in the patient with AML is mainly guided by the patient's fitness of receiving intensive chemotherapy.

Patients considered fit for intensive chemotherapy, receive an induction therapy based primarily on the administration of anthracyclines and cytarabine, or alternatively on FLAG-IDA and mytoxantrone-based cytarabine. Patients resulted mutated for a *FLT3* mutation at diagnosis receive also the kinase inhibitor midostaurin, that is demonstrated to significantly improve the Overall Survival (OS). (29) Newer and potentially more potent *FLT3* inhibitors are under investigation in several clinical trials.

Moreover, other therapeutic options are being evaluated in several clinical trials to better induction treatment. Particularly, an anti-CD33 humanized antibody conjugated with a potent cytotoxic agent (N-acetyl-gammacalicheamicin DMH) is a possible alternative option that demonstrated to improve survival. It is suggested for AML positive for CD33 surface marker with favorable or intermediate risk. (16).

On the other hand, for patients affected by AML therapy related, a history of MDS/MDS or a AML with myelodisplasia-related genetic abnormalities, a liposomal formulation that encapsulates cytarabin/daunorubicin at 5:1 ratio (CPX-351) is suggested in combination with conventional chemotherapy. This combination demonstrated to improve response rate and OS in patients with these characteristics and older than 60 years old. (30)

Consolidation therapy is ideally a further treatment, administered after the CR achievement, to eradicate the remaining leukemic cells and prevent a relapse. The decision to perform an allogeneic stem cell transplantation in AML patients depends on the risk-benefit ratio. The estimation of this ratio is based on the genetic features at diagnosis and response to initial therapy as well as the transplant related factors. The allo-HSCT procedure is

suggested for patients with adverse-risk AML and for the great part of the patients with an intermediate–risk. Lacking of MRD clearance in CR is crucial to referring patients affected by a favorable risk AML to transplantation. (31) In absence of indication to allo-HSCT, the consolidation program is ideally a regimen that include intermediate-dose cytarabine.

The role of maintenance has the goal to minimize the risk of relapse reducing the toxicity at the minimum. Several trials are ongoing to investigate different agents as maintenance therapy to prolong CR in AML patients. (32)

For patients considered unfit for intensive chemotherapy, relevant advances have been made in the last few years. These patients are treated with a low-intensity therapies based on the administration of low dose cytarabine or hypomethylating agents in combination with venetoclax, a BCL-2 inhibitor. In presence of a mutation involving IDH1, the addition of ivosidenib, a IDH inhibitor, to the hypomethylating treatment can be considered. For this reason, the evaluation of IDH1/2 mutation at diagnosis, particularly for older patients, became substantially relevant. (33)

1.2. Myelofibrosis

In 1951, Dameshek coined the term myeloproliferative disorders(34), which, in the following years, was transformed by WHO in myeloproliferative neoplasms (MPNs).

Based on 2001 WHO classification, revised in 2008, classic types of myeloproliferative neoplasms are: Chronic myeloid leukemia (CML), Polycythemia Vera (PV), Essential Thrombocythemia (ET), and Primary

Myelofibrosis (PMF). WHO classification also included Chronic Neutrophilic Leukemia (CNL), Chronic Eosinophilic Leukemia (CEL), and MPN unclassifiable. Classic types of MPNs includes CML which is characterized by the presence of translocation t(9;22) and consequently of the *BCR-ABL1* transcript, while PV, ET, and PMF are *BCR-ABL1* negative(35). The term Chronic Myeloproliferative Neoplasms (MPNs) identifies a heterogeneous group of closely related pathologies that originate from the neoplastic transformation of the pluripotent stem cell leading to an overproduction of both mature and immature cells in one or more cell types of the myeloid lineage (36). These pathologies are characterized by the clonal and uncontrolled proliferation of one or more hematopoietic progenitors in the bone marrow and, in many cases, also in extra-medullary sites, especially in the spleen and liver. The WHO classification published in 2016 for myeloid malignancies included recent advances in hematology with the identification of molecular and prognostic markers, giving a better understanding of the molecular pathogenesis and genetics of the hematological malignancies.

Among MPNs, we can distinguish:

Polycythemia Vera

Polycythemia Vera (PV) is characterized by the increase in the erythrocyte mass, often accompanied also by leukocytosis and thrombocytosis. Histologically, it shows panmyelosis with erythroid, granulocyte, and megakaryocyte hyperplasia. Biologically the disease is characterized by the independence of the hyperplasia from the growth factors erythropoietin(37). The extra blood cells may collect in the spleen causing it

to swell. Furthermore, the increased number of RBCs, WBCs and platelets can cause bleeding problems leading to blood clots forming, and increasing the risk of stroke or heart attack. Patients also have a higher risk to develop acute myeloid leukemia or primary myelofibrosis. (38)

The analysis of 20 studies of PV patients from around the world revealed an annual incidence of 0.84 cases per 100000 people, with no difference between genders(39).

Essential Thrombocythemia (ET)

Essential Thrombocythemia is a disease characterized by thrombocytosis and associated with thrombotic and hemorrhagic complications. Histologically, it is characterized by a medullary picture of marked megakaryocytic hyperplasia with normal erythropoiesis and granulopoiesis (37).

The annual incidence varies from 1 to 2.5 per 100000 and appears slightly more common in females(39).

Myelofibrosis

Myelofibrosis (MF) is a *BCR-ABL1* negative myeloproliferative neoplasm (MPNs), characterized by stem cell-derived clonal proliferation, abnormal cytokine expression, varying degrees of bone marrow fibrosis, extra-medullary hematopoiesis, anemia, leukoerythroblastosis splenomegaly, constitutional symptoms, leukemic progression, and poor survival (40). The primary criteria to differentiate PMF from ET or PV are the presence of hyperplastic and fibrosis, resulting from reticulin and/or collagen fibers

deposition and dysplastic megakaryocytes with maturation defects and bone marrow. Moreover, a peculiar characteristic of MF is the over-production of platelets released into the blood and producing cytokines that stimulate the development of fibrous tissue in the marrow(41). Median survival in MF is estimated at 6 years but can range from months to several years. (42) Patients with MF are at risk for premature death, and their quality of life can be compromised by a severe anemia, marked splenomegaly, profound constitutional symptoms, and cachexia. (43) Causes of death include leukemic transformation, disease progressions without acute transformation, thrombosis, infection, bleeding, and complications of portal hypertension. (44)

MF can develop in two different ways:

1. Primary MF (PMF or Chronic Idiopathic Myelofibrosis) develops “de novo” in patients with no history of previous myeloproliferative disease. It is further sub-classified into “pre-fibrotic” and “overtly fibrotic” PMF, mainly basing on the grade of fibrosis. (45)

2. Secondary myelofibrosis, is characterized by a developing of fibrosis from a previous PV or ET. Usually, 15% of patients with ET or PV develop a PMF-like phenotype over time, referred to as post-ET or post-PV MF. (46,47) The median time to transformation is 7-20 years from PV/ET diagnosis(48), while the reported rates of transformation are 10-15% for PV and 5-10% for ET. (Passamonti et al., 2004b)

The incidence of MF is approximately 0,1 to 1 per 100,000 individuals per year(50). The annual incidence in males is slightly higher than in females (0,59 vs 0.30 p=0.05), with a ratio of 1.6, while no significant difference between geographic regions was observed (0.46 per 100,000 in Europe, 0.46 per 100,000 for North America, and 0063 per 100,000 for Australasia p=0.435)(36).

MF is typically an adult-elderly pathology, usually occurring after age 50 years, with, a median age at diagnosis of 64 years(50). MF is a very rare disease in pediatric population, in which occurs in the first 3 years of life. Familial infantile myelofibrosis mimics the adult disease and in some cases is transmitted by autosomal recessive inheritance.(46)

The median survival was 5 years before 1995(42) and increased to 6.5 years between 1996 and 2007 following the improvement of supportive treatments and earlier diagnosis (50,51) MF is characterized by a very heterogeneous natural outcome, with a global survival ranging from some months to some decades.

1.1.2 Molecular pathogenesis

In MF, chromosomes anomalies are common (about 30% in PMF), but none of those is specific. Indeed, aberrations are very heterogeneous, ranging from gains and losses of genetic material to structural changes including unbalanced translocations. Among those, the most frequent are 9p (trisomy ore partial trisomy), 20q-, 13q-, 8 or 9 trisomy, and partial trisomy of 1q(52). The turning point in understanding MF molecular pathogenesis derived from the new genome-wide sequencing technologies. Since 2005, indeed, different genetic alterations were found outlining the mutational pattern

responsible for the pathogenesis of myelofibrosis (46). The so-called "phenotypic driver mutations", which are those capable of driving the myeloproliferative phenotype, involve *JAK2*, *CALR*, or *MPL* genes. These mutations are mutually exclusive, except for rare cases. (53) Evidences documenting that mutations in *JAK2*, *CALR*, or *MPL* are sufficient to produce an MPN phenotype has been provided by mouse models, where expression of each mutation alone accurately recapitulate distinctive features of human disease(54).

The vast majority of cases of PMF and post-ET MF were characterized by the presence of at least one of these mutated genes, while nearly all cases of post-MF PV are virtually exclusively a *JAK2*-driven disease(55). In particular:

1. *JAK*2V617F mutation, is carried by almost all patients with PV and it occurs in a frequency of 55% in PMF and of 65% in ET;
2. *CALR* indels affect 25% to 35% of PMF patients, 15% to 24% of those with ET(55) ;
3. *MPL* mutations occur in almost 8% and 4% of patients with, , PMF and ET(34) respectively.

In some of these cases non-canonical *JAK2* and *MPL* mutations have been described(55). A 10% of PMF patients lack identifiable driver mutations, but other clonal markers are usually detectable. In addition, non-diver mutations, mostly affecting genes involved in epigenetic modification of chromatin or RNA splicing, are largely described in MF. Mutations in *ASXL1*, *EZH2*, *TET2*, *DNMT3A*, *SRSF2*, *U2AF1* are frequently found at different stages of the disease, and some of those were associated with inferior survival and

have been incorporated into prognostic models. Other non-driver mutations are more common in blast phase than in chronic phase, such as *IDH1/2*(55). To summarize, driver mutations are essential for the development of the MPN phenotype, whereas the non-driver mutations might contribute to disease progression and leukemic transformation(55).

1.2.3 MF Diagnosis

Diagnosis of MF is based on the WHO-2016 criteria, minimally revised in the last version published in 2022. The diagnosis of MF is principally histological, but takes into account also the molecular features of the disease. Molecular driver mutations in *JAK2*, *MPL*, and *CALR* represent a major criterion for establishing the clonality of the disease. In the absence of driver mutations, disease clonality can be demonstrated with one of the epigenetic non-driver mutations, the most common being *ASXL1*, *EZH2*, *TET2*, *IDH1*/*IDH2*, *SRSF2*, *SF3B1.* (56,57) Diagnostic criteria for MF are summarized in Table 6

Primary myelofibrosis (overtly fibrotic)	**Primary myelofibrosis (pre-fibrotic)**
Major Criteria	
Typical megakaryocyte changes, accompanied by ≥ grade 2 reticulin/collagen fibrosis	Typical megakaryocyte changes, accompanied by > grade 1 reticulin/collagen fibrosis
Presence of *JAK2*, *CALR*, or *MPL* mutations, or presence of other clonal markers, or absence of evidence for reactive bone marrow fibrosis	Presence of *JAK2*, *CALR,* or *MPL* mutations, or presence of other clonal markers, or absence of evidence for reactive bone marrow fibrosis
Not meeting WHO criteria for other myeloid neoplasms	Not meeting WHO criteria for other myeloid neoplasms

Minor criteria	
Anemia not otherwise explained	Anemia not otherwise explained
Leukocytosis ≥ 11 × 109/L	Leukocytosis ≥ 11 × 109/L
Palpable splenomegaly	Palpable splenomegaly
Increased serum lactate dehydrogenase	Increased serum lactate dehydrogenase
A leukoerythroblastic blood smear	

Table 6*:* 2016 revised WHO diagnostic criteria for primary myelofibrosis (In both forms diagnosis requires meeting all three major criteria and one minor criterion) (Table adapted by Arber et al., "The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia")

In 2016, the WHO revised criteria provided a further classification of PMF in pre-fibrotic/early primary myelofibrosis (pre-PMF) and overt fibrotic myelofibrosis (overt PMF) basing essentially on the level of bone marrow fibrosis. Different studies underline the difference between the two forms of PMF, both in terms of presentation and outcome. Compared with overt PMF, patients with pre-PMF were generally females of young age who showed a more pronounced myeloproliferative phenotype with higher leukocyte, hemoglobin, and platelet levels. On the contrary, in patients affected by pre-PMF peripheral blood blast, symptoms, and extensive splenomegaly were observed less frequently. (45) In the initial phase, pre-fibrotic PMF, the bone marrow may be hypercellular with prominent granuloblastic and megakaryocyte proliferation and reduction of the erythroid lineage. The aspect that characterizes this phase, in addition to the morphological alterations of the megakaryocytes, is the presence of medullary fibrosis of a grade not exceeding 1 according to the European criteria of gradation, while in the "overt" form the marrow tends to be progressively hypocellulated with a reduction in particular of the erythroid

component and prevalence of megakaryocyte proliferation, with highly abnormal elements under the morphological profile and with a marked tendency to the formation of cohesive clusters (58).

Diagnosis of post-PV or post-ET Myelofibrosis should adhere to criteria published by the International Working Group for MPN Research and Treatment (IWG-MRT)(47), summarized in Table 7.

Post-Polycythemia Vera Myelofibrosis (post-PV MF)	Post-Essential Thrombocythemia myelofibrosis (post-ET MF)
Required criteria	
Prior documentation of WHO-defined PV	Prior documentation of WHO-defined ET
Bone marrow fibrosis grade ≥ 2	Bone marrow fibrosis grade ≥ 2
Additional criteria (two required):	Additional criteria (two required):
Anemia or loss of phlebotomy requirement	Anemia and ≥ 2 g/dL decreases in hemoglobin level
A leukoerythroblastic blood smear	A leukoerythroblastic blood smear
Increasing splenomegaly	Increasing splenomegaly
Development of constitutional symptoms	Development of constitutional symptoms Increased serum lactate dehydrogenase

Table 7 2016 revised WHO diagnostic criteria for primary myelofibrosis (In both forms diagnosis requires meeting all three major criteria and one minor criterion) (Table adapted by Arber et al., "The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia")

1.2.4. Clinical aspect related to MF and evolution to leukemia

The symptoms of myelofibrosis can be classified into 3 main categories: myeloproliferative, cytopenic, and constitutional. The first group includes hepatosplenomegaly, which may represent the most important symptom for the patient. Massive splenomegaly can be painful, compress and dislocate the abdominal organs and favor the appearance of ascites and pleural and pericardial effusions, as well as facilitate splenic infarcts and/or abdominal thrombosis. (43,46) Cytopenias of varying severity are characteristic of myelofibrosis and are multifactorial. Anemia is the most common cytopenia and can have various causes which include ineffective hematopoiesis, splenic sequestration, and hemolysis. (46). Thrombocytopenia can worsen bleeding risk, while leukopenia is rarer and rarely marked. More frequently, leukocytosis is observed with a typical leukoerythroblastic pattern, characterized by immature elements of the granuloblastic series in the peripheral blood and by erythrocyte anisopoikilocytosis with "drop" cells, or dacryocytes(43,46). The high cell turnover and extramedullary hematopoiesis typical of the disease can also induce a hypercatabolic state with constitutional symptoms, even severe ones, such as weight loss, asthenia, fever, and cachexia(59).

Transformation of MF into acute leukemia occurs as a terminal event in a minority of patients. The evolution into a blast phase (defined by the presence of ≥20% blasts in the blood or bone marrow) is a risk in all the MPN variants, but the incidence is higher in MF compared to the other MPN, accounting for 10% to 20% of patients in the first 10 years of disease(60,61). Genomic and functional analyses of post-MPN acute myeloid leukemia

(AML) samples have revealed a mutational profile that is quite distinct from that of de novo AML. In addition, , *IDH1/2* or *TP53* mutations are uncommon in chronic-phase MPN and substantially more frequent in blast phase(55). The dismal prognosis of AML secondary to a MF has also been highlighted by recent study in which specific treatment did not appear to influence either survival whereas durable remissions have been noted in few patients receiving allogeneic stem cell transplant (62).

1.2.5. Prognosis and prognostic score

MF patients are characterized by a huge variability in prognosis. In some cases, patients affected by MF may have a mild form of the disease that doesn't progress rapidly, whereas others may progress more quickly and requires regular blood transfusions or drug treatments and a high risk to progress to a blast form. Different prognostic models have been developed over the time to evaluate the MF prognosis and plan a risk-adapted treatment strategy for the patient.

The most commonly used score in clinical practice, based exclusively on cynical-hematological variables easily accessible to the clinician, is the International Prognostic Scoring System (IPSS). Since the IPSS is calculated using data obtained at diagnosis, Dynamic International Prognostic Scoring System (DIPSS) was developed to recalculate the patient's risk category at each time of follow-up. Other prognostically significant clinical parameters (need for transfusion, platelet count <100 x 10^9/L, and an unfavorable karyotype) were incorporated into the first clinical-genetic mixed score, called DIPSS-plus.

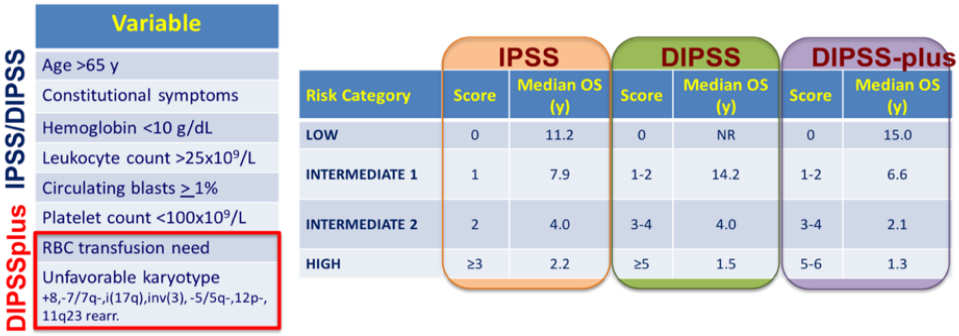


Figure 2: PSS, DIPSS and DIPSS-pls prognostic systems for patients with PMF. (Picture taken from "Sindromi mieloproliferative croniche", Barbui & Vannucchi. Ematologia in progress)

Figure 2 shows variables with a prognostic significance considered for the calculation of clinical-based score, IPSS, DIPSS and DIPSS-plus.

Following the discovery of the presence of additional non-driver mutations in MF patients, in 2013 a large cohort of MF patients was studied to determine the prognostic relevance of these somatic mutations. (63) Blood samples collected at the time of diagnosis were analyzed for mutations in *ASXL1*, *SRSF2*, *EZH2*, *TET2*, *DNMT3A*, *CBL*, *IDH1*, *IDH2*, *MPL,* and *JAK2*. Of these, *ASXL1*, *SRSF2*, and *EZH2* mutations independently predicted reduced survival. Further analysis of 797 patients with primary MF then evaluated the effect on Overall Survival (OS) of a limited series of "prognostically unfavorable" mutations in 5 genes (*ASXL1*, *EZH2*, *SRSF2*, and *IDH1/IDH2*). Median survival was 12.3, 7.0, and 2.6 years, respectively, for those without, with one, or with two or more mutations in these five genes. (64)

Scoring System	Factors (point)	Risk groups scores
MIPSS70	- Hb < 100 g/L (1) - WCC > 25 X 10^9/L (2) - PB blasts ≥ 2% (1) - Plt < 100 X 10^9/L (2) - Constitutional symptoms (1) - Bone marrow fibrosis grade ≥ 2 BM (1) - Absence of CALR Type 1 (1) - One HMR mutations (1) - ≥ 2 HMR mutations (2)	Low risk: 0-1 points (not reached) Intermediate risk: 2–4 points (6.3y) High risk : ≥5 points (3.1y)
MIPSS70+	- Severe anemia (2) - Moderate anemia (1) - PB blasts ≥ 2% (1) - Constitutional symptoms (2) - VHR karyotype (4) - Unfavorable karyotype (3) - Absence of CALR Type 1 (2) - One HMR mutations (2) - ≥ 2 HMR mutations (3)	very low risk: 0 points (not reached) Low risk: 1-2 points (16.4y) Intermediate risk: 3–4 points (7.7y) High risk: 5-8 points (4.1y) very high risk: ≥9 points (1.8y)
GPSS	- VHr karyotype (2) - Unfavorable karyotype (1) - Absence of CALR Type 1 (1) - ASXL1 mutation (1) - SRSF2 mutation (1) - U2AF1 mutation (1)	Low risk: 0 (26.4y) Intermediate-1: 1 (8y) Intermediate-2: 2 (4.2y) High risk: ≥3 (2y)

Table 8: Comparison between different prognostic scoring systems. WCC, white cell (leukocytes) count; y, years.

Based on these results, the first score to integrate mutations and clinical variables was developed. The “Mutation-Enhanced IPSS” (MIPSS70) take

into account 9 variables, including 3 genetics and 6 clinical risk factors, these variables are described in Table 8. The sum of these scores characterizes a low (0-1), intermediate (2-4), high (≥5) risk categories characterized by median survival ranges of 27.7 years “not reached”, 6.3-7.1 years, and 2.3-3.1 years. (65) MIPSS70-plus integrated mutations, +9, 20q-, chromosome 1 translocation/duplication or sex chromosome clinical variables, and al karyotype, applying the new cytogenetic risk stratification, proposed by Tefferi in 2018, based on three groups (66):

- very high risk (VHR): containing single/multiple abnormalities of -7, i(17q), inv(3)/3q21,12p-/12p11.2,11q-/11q23, or other autosomal trisomies not including +8/ +9;
- favorable: containing normal karyotype or sole abnormalities of 13q-, abnormality including –Y;
- unfavorable: all other abnormalities.

MIPSS plus considers also U1AF1Q157 as an additional high molecular risk mutation, and new sex- and severity-adjusted hemoglobin thresholds. It includes five genetics and four clinical risk factors and stratifies patients in five risk categories: vary low risk (0), low risk (1-2), intermediate-risk (3-4), high risk (5-8), and vary high risk (≥ 9). In the cohort of patients, the corresponding median survivals were respectively: not reached, 16.4, 7.7, 4.1, and 1.8. (67) Figure 3 shows the stratification of the patients in the study conducted by Guglielmelli et al according to MIPSS scoring systems.

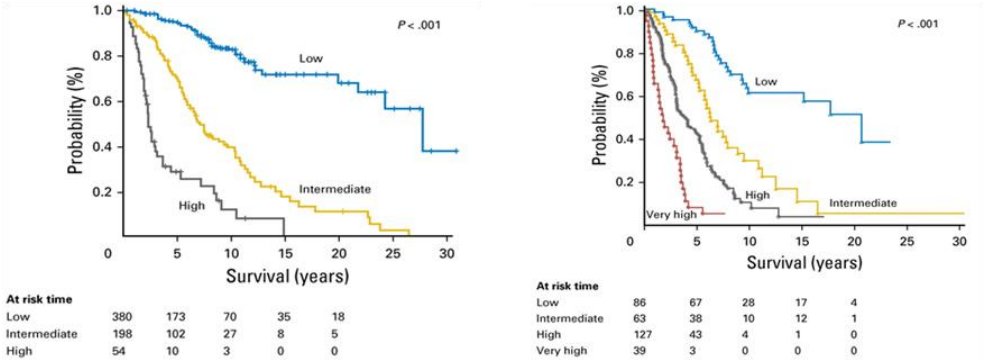


Figure 3 Survival curve of patient classified using a MIPSS70 prognostic scoring system (left) and MIPSS70-plus (right), (Picture A taken from "MIPSS701 version 2.0: Mutation and karyotype-enhanced international prognostic scoring system for primary myelofibrosis", Tefferi et al; Picture B taken from "MIPSS70: Mutation-Enhanced International Prognostic Score System for Transplantation-Age Patients With Primary Myelofibrosis",Guglielmelli et al 2018, JCO)3:

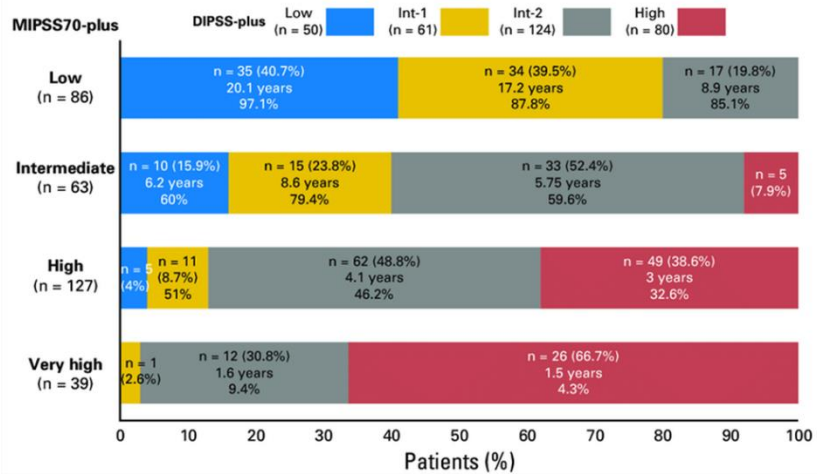


Figure 4: Categorization of patients according MIPSS70-plus versus DIPSS-plus (Picture A taken from "MIPSS70: Mutation-Enhanced International Prognostic Score System for Transplantation-Age Patients With Primary Myelofibrosis",Guglielmelli et al 2018, JCO)

Guglielmelli et al, in 2018 compared the distribution of patients in the MIPSS70-plus with DIPSS-plus risk categorization. Figure 4 shows that there is a significant risk redistribution based on the used scoring system. (65)

GIPSS offers a low-complexity risk model for MF stratification based exclusively on karyotype data and a limited number of mutations, involving *ASXL1*, *SRSF2*, *U2AF1*, and *CALR*. This scoring system is solely dependent on genetic risk factors, but does not consider any clinical features.(68)

1.2.6. MF Therapy

The only treatment capable of eradicating MF is allogeneic hematopoietic stem cells transplantation (allo-HSCT). However, this procedure is still characterized by important mortality rate regardless the intensity of conditioning regimen and, thus, need to be reserved only to a minority of patients. (69)(69) For this reason, it is important to have reliable prognostic models that facilitate treatment decision making. (70) and that allow accurate risk stratification of patients in term of overall and leukemia-free survival, as previously described.

1.2.6.1 Symptomatic treatments

The available treatments for patients affected by MF are mostly oriented to manage the symptoms associated to the disease. Figure 5 shows an algorithm that suggests the best treatment choice for MF patients on the basis of biologic features.

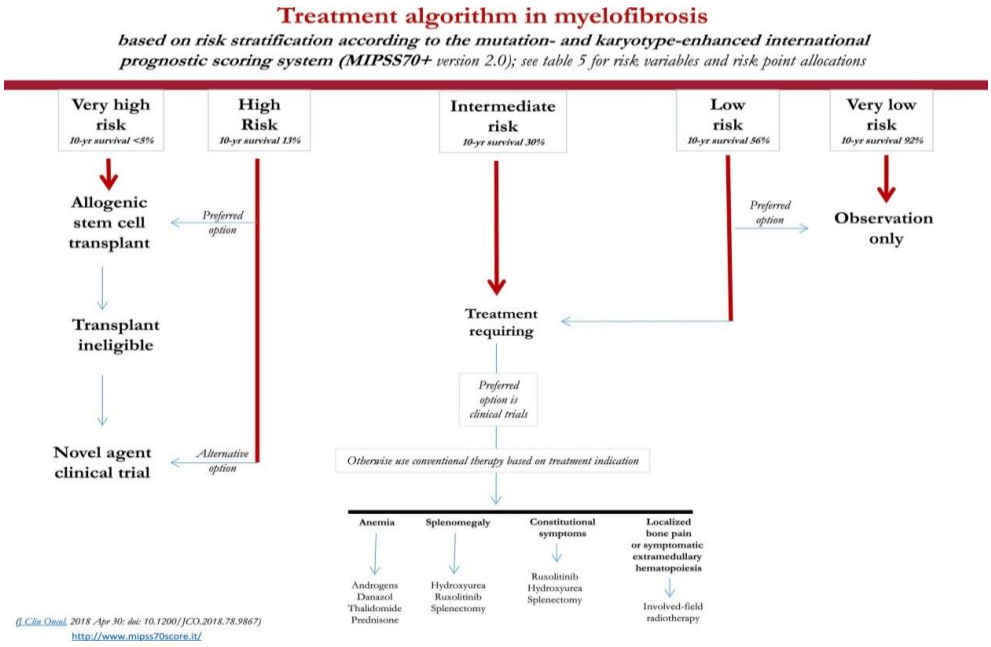


Figure 5: Treatment algorithm in myelofibrosis based on risk stratification according to the mutation- and karyotype-enhanced international prognostic scoring system. (Picture taken from: "MIPSS701 version 2.0: Mutation and karyotype-enhanced international prognostic scoring system for primary myelofibrosis", Tefferi 2018, JCO)

Suggested treatments for MF-related anemia are: corticosteroids, dazol, erythropoietin, thalidomide, and lenalidomide, also being treated together. Each of them has limitations and there are no comparative clinical studies that allow the best treatment to be established. Severe anemia requires transfusion (46), resulting in iron overload(70), reducible by iron chelation.

Symptomatic splenomegaly in myelofibrosis occurs in 10% of cases at diagnosis but develops in more than 50% of patients over the course of the disease(46). Drugs used to reduce massive splenomegaly are hydroxyurea, melphalan, and busulfan, but the results were unsatisfactory. Splenectomy

is an option to consider in cases of massive splenomegaly resistance to medical therapy, including *JAK2*-inhibitors, if associated with symptomatic portal hypertension (bleeding varices, ascites), splenic pain, cachexia, or frequent need for transfusion (71) Hematopoietic recovery seems to be influenced by spleen size at transplantation. In a protocol performed by GITMO patients that underwent previous splenectomy had a significantly faster neutrophil and platelet recovery, compared with patients with splenomegaly before allo-HSCT, which had slower neutrophil and platelet engraftment(72).

The treatment with *JAK2* inhibitors is suggested for splenomegaly and for the mitigation of the other constitutional symptoms. The discovery of the *JAK2* mutation and the deregulation of the JAK-STAT pathway regardless of the type of driver mutation in MF has allowed the development of small molecules inhibiting tyrosine kinases. These drugs act mainly by inhibiting the deregulated JAK-STAT pathway which is altered in all patients with MF.

Ruxolitinib is an oral inhibitor that has been approved on the basis of the results obtained by two randomized phase III studies that compared the drug against placebo (COMFORT-1) or best available therapy (BAT) prescribed by European participating centers (COMFORT-2). The two trial enrolled patients with PMF or SMF classified as intermediate/high risk (based on IPSS)(73,74). The results of the two trials showed that a higher proportion of patients treated with the drug ruxolitinib achieved the primary endpoint, which was at least a 35% reduction in splenomegaly after 24 weeks in COMFORT-1 and 48 weeks in COMFORT-2. (75). Of great interest is the data on the improvement in OS that emerged from the prolongation of

the observation of patients enrolled in the two clinical trials. (76). The drug was well tolerated and has shown efficacy on systemic symptoms (sweating, itching, asthenia) which regressed within 4-6 weeks, allowing for a significant improvement in the quality of life.

The median duration of response to ruxolitinib is 3-5 years. The loss of response is associated with a markedly reduced survival and refers to the acquisition of clonal progression, i.e. the appearance of new mutations and/or the progressive increase in the allelic charge of the pre-existing ones. (77) Other *JAK*-inhibitors are being studied in clinical trials, including fedratinib, momelotinib, pacritinib.

Lastly, interferon α is an effective drug used for the treatment of PV and ET, controlling the symptoms mainly through cytoreduction. However, MF is a far more advanced disease characterized by additional non-driver mutations, which are described to be associated to a low rate of response to treatment. (78) Furthermore, toxicity such as cytopenia is more common in advanced MF than in PV and ET, leading to more frequent discontinuation. For these reasons, the treatment of MF with interferon α is limited and combinations with other medications are being developed to enhance the poor effect of IFNα in patients with MF and to improve the tolerability.

1.2.5.2. Allogeneic Hematopoietic Stem Cell Transplant (allo-HSCT)

Hematopoietic Stem Cell Transplantation (HSCT) is the only curative approach in patients with MF. Several improvements, such as less toxic conditioning regimen, better HLA donor selection and anti-infective therapies, have been introduced in the last years. These advances in

supportive care and the application of RIC regiments have widened the rage of people that can undergo allo-HSCT to elderly and medically unfit patients(79). However, the procedure is still related with significant relapse and non relapse mortality), (80) even because, in patients affected by MF, HSCT is complicated by a high incidence of poor graft function or graft failure caused by both splenomegaly and by a “pro-inflammatory” marrow niche characterizing MF biology.

To optimize both the indications and the procedures of HSCT in MF, a consensus process has recently been completed between experts in EBMT and ELN. Transplantation up to 70 years has been indicated in intermediate-2 or high-risk risks. Cases up to 65 years are candidates if classified as intermediate-1 as long as they have a dependence on transfusions, peripheral blasts greater than 2%, or unfavorable cytogenetics. (81) The integration of these clinical scores with the molecular characteristics in the MIPSS-70 scores can be useful to address MF patients to HSCT. It is particularly relevant for those patients traditionally categorized as low- or intermediate-1 risk, although further validation is required, because limited data are available regarding the impact of somatic mutations on transplantation outcomes in MF. (82)

More recently, a prediction score for the risk of mortality associated with the transplant procedure, MTSS, has been proposed. (83)

Comprehensive clinical-molecular transplant scoring system for MF undergoing HSCT (MTSS).

	HR (95% CI)	P	weighted score	Our pt
age ≥ 57 years	1.65 (1.15-2.36)	0.006	1	0
Karnofsky performance status <90%	1.50 (1.06-2.13)	0.021	1	0
non-CALR/MPL driver mutation genotype	2.40 (1.30-4.71)	0.012	2	2
*ASXL1*mutation	1.42 (1.01-2.01)	0.041	1	1
HLA-mismatch unrelated donor	2.08 (1.45-2.97)	<0.001	2	0
WB count >25x10^9/L	1.57 (1.16-2.41	0.007	1	1
Platelet count <150x10^9/L	1.67 (1.16-2.40)	0.006	1	0

LR = 0-2
IR = 3-4
HR = 5
vHR=>5

MTTS= Int R

Figure 6: Transplant scoring system for MF undergoing HSCT. (Picture taken from "Sindromi mieloproliferative croniche", Barbui & Vannucchi. Ematologia in progress

At present, standard conditioning regimen or prophylaxis for graft-versus-host disease (GVHD) have not been defined yet. A direct comparison of RIC and MAC regimens is extremely difficult but a large retrospective analysis of the EBMT including 2224 patients with MF, stratified according to conditioning intensity, was performed. This study showed no statistically significant differences in terms of engraftment, GvHD, NRM, and overall survival, when the 2 regiment were compared. On the contrary, a higher relapse rate in patients receiving the RIC regimen was observed.

1.3. Next Generation Sequencing (NGS) and its application in risk classification

NGS or parallel massively sequencing is a biologic technology that allows the simultaneous sequencing of millions of fragments of DNA or complementary DNA (cDNA synthetized starting from RNA molecules). NGS became commercially available in 2005 and has been rapidly adopted in the clinical laboratory thanks to its ability to simultaneously analyze several genes or gene regions from different samples by a single test. This technique demonstrated to be more advantageous compared to traditional Sanger sequencing by capillary electrophoresis which allow to sequence one DNA fragment at a time.(84). NGS in the clinical laboratory has evolved and will continue to evolve over time with new applications adapting to increasing clinical needs and providing ideal throughput per run performable quickly and cost-effectively. NGS enables the discovery and analysis of different types of genomic features (i.e. single nucleotide variants, copy number, structural variants) in a single sequencing run starting from a low amount of sample input. For these reasons, NGS has a wide spectrum of application in laboratory medicine and has become an integrated part of precision medicine to study genetic variation associated to diseases or to other biologic characteristics. In particular, the technology has been used in diagnosis, prognosis, and therapy selection for constitutional disorders, oncology and infectious diseases. (85)Since cancer is a genomic disease, the identification of genomic aberrations in cancers has become an integral part of oncology. Although the availability of whole genome, exome, or transcriptome sequencing has been increasing, targeted gene sequencing is the method of choice in clinical laboratories for cancer diagnosis ensuring

optimal sequencing quality (read depth and coverage, variant characterization, reporting), cost-effectiveness, and turnaround time.

As widely described in this introductive chapter, current diagnosis of myeloid malignancies has been rapidly evolving also thanks to the introduction of NGS in specialized hematology laboratories which apply for clinical purpose commercially available gene panels for targeted resequencing. (86) With the help of myeloid gene panels application, recurrent somatic mutation can be identified in most AML patients, and, even within defined AML entities, additional molecular genetic mutations are detectable in many cases. This characterization by sequencing allows the classification in new molecularly-defined entities by WHO and definition of risk as recently proposed (ELN). Moreover, the molecular landscape is also investigated in MPNs. Besides the driver mutations involving *JAK2*, *CALR*, and *MPL*, non-driver mutations in the genes known from MDS and AML are also detected in PV, ET and MF (87,88) to identify patients that are at higher risk for progression and transformation by applying molecular scoring systems.

Challenge	Background	Current and future approaches

Discrimination of leukemia-related mutations from polymorphisms or passenger mutations	Driver mutations expected to occur at higher allele frequency in patient samples than passenger mutations; driver mutations more likely to have an impact on protein function than polymorphisms or passenger mutations	-Optimization of cancer-specific databases including reporting of rare physiological gene variants. -Implementation of novel bioinformatic algorithms based on prediction of functional impact -Quantitative and dynamic VAF monitoring (separately and together with other mutations) at follow-up
Discrimination of somatic leukemia-related mutations from CHIP	CHIP is presented in about 10% of individuals aged 70 to 80 and in up to 20% in the age group > 80 years	-Quantitative and dynamic VAF monitoring (separately and together with other mutations) at follow-up. -Clarifying the significance of CHIP in myeloid malignancies
Discrimination of leukemia-related somatic mutations from pathogenic germline alterations	Need to differentiate acquired somatic mutations from germline pathogenic variants at diagnosis	-Mutation detection in germline control samples (e.g., skin fibroblasts, saliva) in mutations such as in RUNX1, CEBPA. -Thorough medical family history followed by molecular genetic tests in relatives if necessary -Evaluation of VAF at follow-up in relation with clinical response to therapy.
Discrimination of true genetic alterations from PCR, sequencing and post-sequencing artifacts derived from technical limits	Many artifacts can arise during NGS library preparation, sequencing and data analysis	-Error correction using molecular identifiers that individually label original input DNA molecules - Refinement of error-correction computational methods in post-sequencing NGS data analysis. -Confirmation using alternative approaches
Limited sensitivity of NGS for minimal residual disease (MRD) assessment	Mutations detected at diagnosis may be re-identified at best to a VAF of 1–2%	Error-corrected sequencing using molecular identifiers.
High financial burden; demand on interdisciplinary approaches	Expensive technical and staff equipment, sophisticated data interpretation. Complex translation of NGS results into therapeutic decision	Development of continuously updated NGS interpretation sets and algorithms for well-established mutational profiles within distinct hematological malignancies Interdisciplinary leukemia boards

Table 10: Open issues in application of NGS in myeloid malignancies evaluation

Inevitably accompanying these developments, some challenges regarding the interpretation and implementation of molecular findings derived from NGS in myeloid neoplasms have emerged over time. The table above (Table 10) is a summary of the open issues in NGS application in myeloid malignancies as suggested by Baker U. et al. (89) NGS has surely opened new horizons for individualized diagnostics and therapy of myeloid malignancies. The application for basic research of expanded panel rather than whole exome or genome sequencing for the characterization of myeloid malignancies will improve our knowledge on these neoplasms and widen the marker to be consider for clinical purposes.

Moreover, molecular MRD monitoring by NGS undergo an expansion in the future as markers suitable for NGS analysis can be identified in virtually all AML patients. The possibility of discordant dynamics of simultaneous mutations requires comprehensive MRD able to monitor different responses to therapies of a polyclonal disease in which some clone could be responsive while other refractory.

While new technological advances may improve the sensitivity and accuracy of NGS-based analyses, its results also deserve cautious interpretation considering the clinical context. For all these reasons, hematologists and pathologists should remain in close interaction with laboratory specialists to avoid misinterpretation of results. Combined efforts will need to optimize the use of NGS techniques in order to guarantee the best management for patients affected by a myeloid malignancy.

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1.5 Aim of the study

Myeloid malignancies are a heterogeneous group of diseases characterized by a common myeloid clonal origin. The WHO classification allow to classify these neoplasms in primary type basing on clinical features combined with morphology, cytochemistry, immunophenotype and genetic characteristics. However, in every single group of diseases the clinical course could be heterogeneous and not easily predictable. This is particularly actual for AML and for MF for which is the greatest clinical risk is represented by the evolution to AML.

Recently, the identification of molecular mutations has dramatically improved our knowledge of AML and MF molecular genetics and shed new light not only on the molecular pathogenesis of the disease but also on the prognostic significance of each mutation and their combination. By applying high throughput sequencing, a better classification of this disease and its prognostic profile is feasible in an adequate timing to make decision regarding the patients' treatment.

The aim of this work Is to better define the role of genes alteration to predict the clinical course of the disease and to select the better clinical strategy in different disease settings.

In particular, each chapter have a clear goal:

Chapter 2: To describe the clinical impact of the gene mutation profile of NK AML patients, belonging to the broad intermediate prognostic subgroup, treated within the prospective NILG trial 02/06

Chapter 3: To investigate the clinical significance of the presence of chromatine-splicesome (CS) mutations in a large cohort of newly diagnosed AML patients enrolled into a prospective trial to identify a subgroup of patients characterized by a clinical outcome similar to secondary-AML

Chapter 4: To evaluate the post-transplant outcome according to the MIPSS70 scores, that include the presence of HMR mutations for calculation, of patients enrolled in a GITMO perspective clinical trial

CHAPTER 2

HIGH THROUGHPUT MOLECULAR CHARACTERIZATION OF NORMAL KARYOTYPE ACUTE MYELOID LEUKEMIA IN THE CONTEXT OF THE PROSPECTIVE TRIAL 02/06 OF THE NORTHERN ITALY LEUKEMIA GROUP (NILG)

High Throughput Molecular Characterization of Normal Karyotype Acute Myeloid Leukemia in the Context of The Prospective Trial 02/06 of the Northern Italy Leukemia Group (NILG)

Salmoiraghi S[1], Cavagna R[1], Zanghì P[1], Pavoni C[1], Michelato A[1] , Buklijas K[1], Elidi L[1], Intermesoli T[1], Lussana F[1], Oldani E[1] , Caprioli C[1], Stefanoni P[1], Gianfaldoni G[2], Audisio E, Terruzzi E, De Paoli L, Borlenghi E, Cavattoni I, Mattei D, Scattolin AM, Taiana M, Ciceri F, Todisco E, Campiotti L, Corradini P, Fracchiolla N , Bassan R, Rambaldi A[1,] and Spinelli O[1].

[1]ASST Ospedale Papa Giovanni XXIII, Bergamo; [2]Azienda Ospedaliera Universitaria Careggi, Firenze; [3] A.O.U. Città della Salute e della Scienza di Torino, Torino; [4]Azienda Ospedaliera San Gerardo, Monza; [5]Azienda Ospedaliera SS. Antonio e Biagio e Cesare Arrigo, Alessandria; [6]ASST-Spedali Civili, Brescia; [7]Ospedale S. Maurizio, Bolzano; [8]ASST Ospedale di Cremona, Cremona; [9]Ospedale dell'Angelo and SS. Giovanni e Paolo, Venezia Mestre; [10]Azienda Ospedaliera S.Croce e Carle di Cuneo, Cuneo; [11]Fondazione IRCCS Istituto Nazionale dei Tumori, Milano; [12]University of Insubria, Varese; [13]IRCSS Ospedale San Raffaele,Milano; [14]IRCCS Istituto Clinico Humanitas di Rozzano, Rozzano; [15]Fondazione IRCCS Ca' Granda Ospedale Maggiore

Policlinico, Milano; [16]University of Perugia, Perugia; [17]University of Milan, Milano

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2.1 Abstract

By way of a Next-Generation Sequencing NGS high throughput approach, we defined the mutational profile in a cohort of 221 normal karyotype acute myeloid leukemia (NK-AML) enrolled into a prospective randomized clinical trial, designed to evaluate an intensified chemotherapy program for remission induction. *NPM1*, *DNMT3A,* and *FLT3*-ITD were the most frequently mutated genes while *DNMT3A*, *FLT3*, *IDH1*, *PTPN11*, and *RAD21* mutations were more common in the *NPM1* mutated patients ($p <$ 0.05). *IDH1* R132H mutation was strictly associated with *NPM1* mutation and mutually exclusive with *RUNX1* and *ASXL1*. In the whole cohort of NK-AML, no matter the induction chemotherapy used, by multivariate analysis, the achievement of complete remission was negatively affected by the *SRSF2* mutation. Alterations of *FLT3* (*FLT3*-ITD) and *U2AF1* were associated with a worse overall and disease-free survival ($p <$ 0.05). *FLT3*-ITD positive patients who proceeded to alloHSCT had a survival probability similar to *FLT3*-ITD negative patients and the transplant outcome was no different when comparing high and low-AR-*FLT3*-ITD subgroups in terms of both OS and DFS. In conclusion, a comprehensive molecular profile for NK-AML allows for the identification of genetic lesions associated to different clinical outcomes and the selection of the most appropriate and effective treatment strategies, including stem cell transplantation and targeted therapies.

2.2 Introduction

Cytogenetic analysis has proved to be crucial for the prognostic stratification of acute myeloid leukemia (AML) patients [1]. However, nearly half of AML patients have a normal karyotype (NK). The identification of molecular mutations has dramatically improved our knowledge of AML molecular genetics and shed new light not only on the molecular pathogenesis of the disease but also on the prognostic significance of each mutation and their combination in NK-AML [2,3]. *NPM1* mutations are found in approximately one third of AML and in about 50% of cases with a normal karyotype [1,4,5]. Alterations involving *NPM1* often occur in combination with other genetic aberrations, which may contribute to determining the disease evolution [3]. Moreover, about 30% of NK-AML [6] is affected by *FLT3*-internal tandem duplication (ITD) resulting in the deregulation of *flt3* kinase activity and determining a worse clinical outcome, even in the presence of *NPM1* mutations [7,8]. Particularly, the evaluation of the *FLT3* allelic ratio (AR) has been included in the European leukemia net (ELN) classification to further improve risk stratification in *FLT3*-ITD mutated AML patients [1], even if this remains a matter of debate [9]. The molecular characterization of AML, obtained by the application of high throughput sequencing, has led to a better classification of this disease and its prognostic profile [1,10]. However, most NK-AML belong to the broad intermediate prognostic subgroup in which the most appropriate treatment strategy remains to be defined. This seems particularly relevant when considering the new drugs targeting specific mutations [11] and the benefit potentially gained by

allogeneic transplantation as post remission consolidation treatment in these patients.

In this context, the purpose of this study was to define the association of molecular mutations with the outcome of a cohort of 221 NK-AML patients treated according to a prospective trial comparing a standard vs. high-dose chemotherapy regimen for remission induction (ClinicalTrials.gov identifier: NCT00495287) [12].

2.3 Results

2.3.1 Clinical and Molecular Findings

The clinical characteristics of the 221 NK-AML patients included in this analysis are summarized in Table 1. The median age at diagnosis was 52 years (range, 19–74 years) and the majority of them (88%) had a de novo AML. The clinical and biological patient characteristics were generally well balanced between the induction arms of the study. (Table 1)

Patient characteristics and mutations	All patients, N=221	ICE, N=117	sHD, N=104	P
Median age, at diagnosis (range)	52.5 (19.8-74.8)	54.4 (23.6-74.8)	49.5 (19.8-72.2)	0.0324
≤ 60 years	166 (75.1)	81 (69.2)	85 (81.7)	0.0319
>60 years	55 (24.9)	36 (30.8)	19 (18.3)	
Sex				0.1765
Female	119 (53.8)	58 (49.6)	61 (58.7)	
Male	102 (46.2)	59 (50.4)	43 (41.3)	
AML category				0.0463
Non de novo	26 (11.8)	9 (7.7)	17 (16.3)	
De novo	195 (88.2)	108 (92.3)	87 (83.7)	

ECOG PS				0.4556
0-1	201 (91)	108 (92.3)	93 (89.4)	
2-3	20 (9)	9 (7.7)	11 (10.6)	
Hepatomegaly	17 (7.7)	8 (6.8)	9 (8.7)	0.6130
Splenomegaly	20 (9)	9 (7.7)	11 (10.6)	0.4556
Extramedullary involvement	34 (15.4)	16 (13.7)	18 (17.3)	0.4550
WBC count (x10^9/L)				0.3677
≤ 50	155 (70.1)	79 (67.5)	76 (73.1)	
> 50	66 (29.9)	38 (32.5)	28 (26.9)	
Hemoglobin (g/dL)	9.5 (4.3-14.1)	9.5 (5.1-14.1)	9.5 (4.3-13.9)	0.9144
Platelets(x10^9/L)	59 (5-815)	64 (5-815)	57.5 (8-513)	0.8752
Bone marrow blast cells, %	80 (0-100)	83 (10-100)	80 (0-100)	0.4519
Peripheral blood blasts cells, %	52 (0-100)	50 (0-100)	55.5 (0-100)	0.6909
Consolidation*				0.3276
No alloHSCT	119 (67.9)	60 (59.4)	59 (66.3)	
alloHSCT	71 (32.1)	41 (40.6)	30 (33.7)	
***FLT3*-ITDwt, *NPM1* wt**	90/216 (41.7)	42/112 (37.5)	48/104 (46.2)	0.1974
***FLT3*-ITD low ratio, *NPM1* wt**	6/221 (2.7)	6/117 (5.1)	0/104 (0)	0.0307
***FLT3*-ITD high ratio, *NPM1* wt**	8/221 (3.6)	4/117 (3.4)	4/104 (3.8)	1.0000
***FLT3*-ITDwt, *NPM1* +**	66/221 (29.9)	34/117 (29.1)	32/104 (30.8)	0.7817
***FLT3*-ITD low ratio, *NPM1* +**	15/221 (6.8)	10/117 (8.5)	5/104 (4.8)	0.2700
***FLT3*-ITD high ratio, *NPM1* +**	31/221 (14)	16/117 (13.7)	15/104 (14.4)	0.8730

Table 1. Patients characteristics according to induction treatment.

According to trial indications [12], 71 out of 190 molecular profiled patients in first complete remission (CR) underwent alloHSCT (Figure 1).

The NGS analysis of the 221 patients identified a total of 738 mutations, including non-synonymous point mutations (missense (*n* = 334) and nonsense (*n* = 42)), insertions or deletions (indels) (in frame (*n* = 112) or causing a frameshift (*n* = 226)), and splicing sites mutations (*n* = 24). The number of molecular alterations per patient ranged from 0 to a maximum of 15, with a median of 3. Only five patients did not present mutations detectable by the applied gene panel. The mutation frequencies according to induction treatment are reported in **Figure 2**, whereas the number of alterations per patient and per gene are represented in **Figure 3**. Moreover,

we measured the association between mutations in different genes, considering genes in pairs (**Figure 4**).

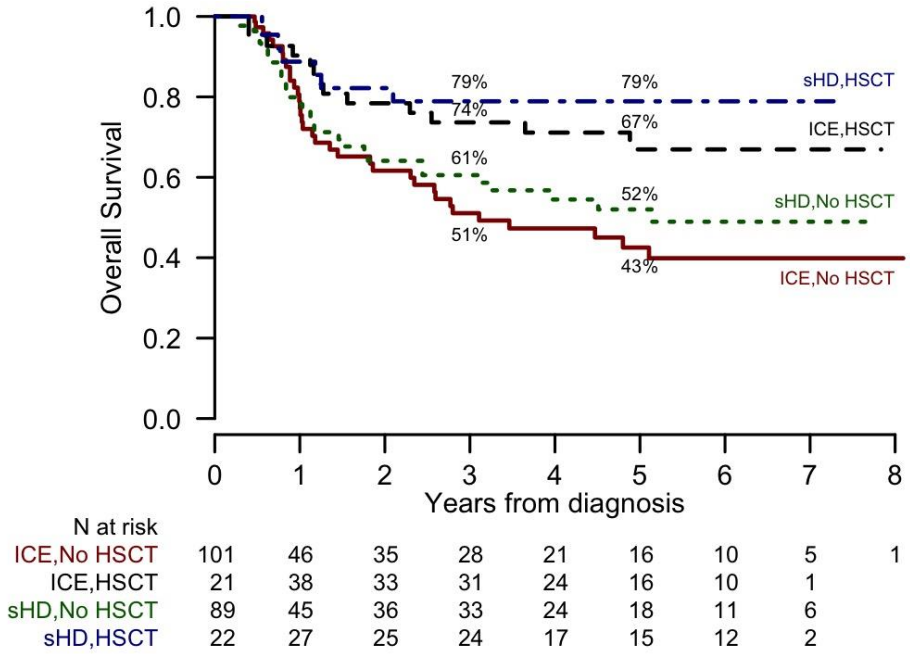


Figure 1. Kaplan-Meier curves of Overall Survival (OS), according to induction and consolidation treatments, in complete remission patients. 5-year OS estimates are reported. *p* values assessed comparing groups are: HSCT, sHD vs. ICE: $p = 0.48$; No HSCT, sHD vs. ICE: $p = 0.52$; sHD, HSCT vs. no HSCT: $p = 0.03$; ICE, HSCT vs. no HSCT: $p = 0.01$.

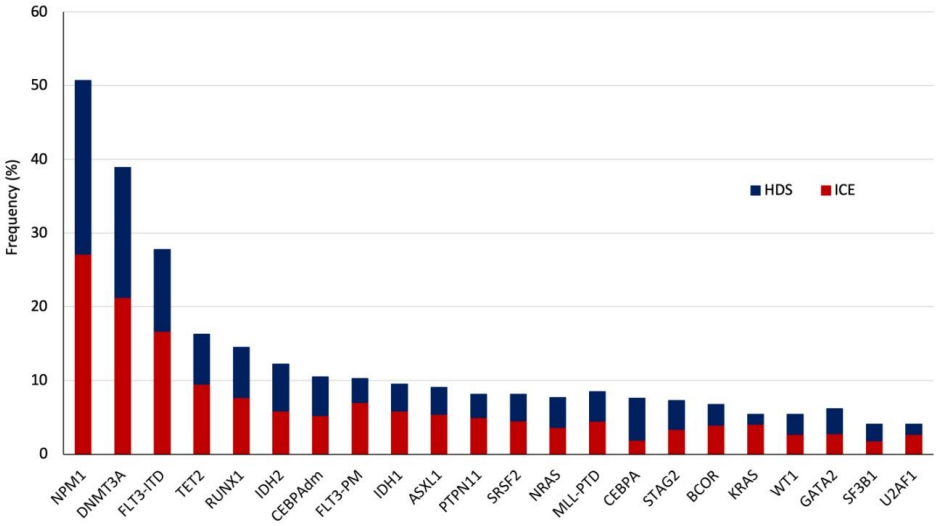


Figure 2: Frequency of different mutated genes according to induction treatment. *CEBPA*2 and *CEBPA*1 indicate the presence of double or single mutation, respectively.

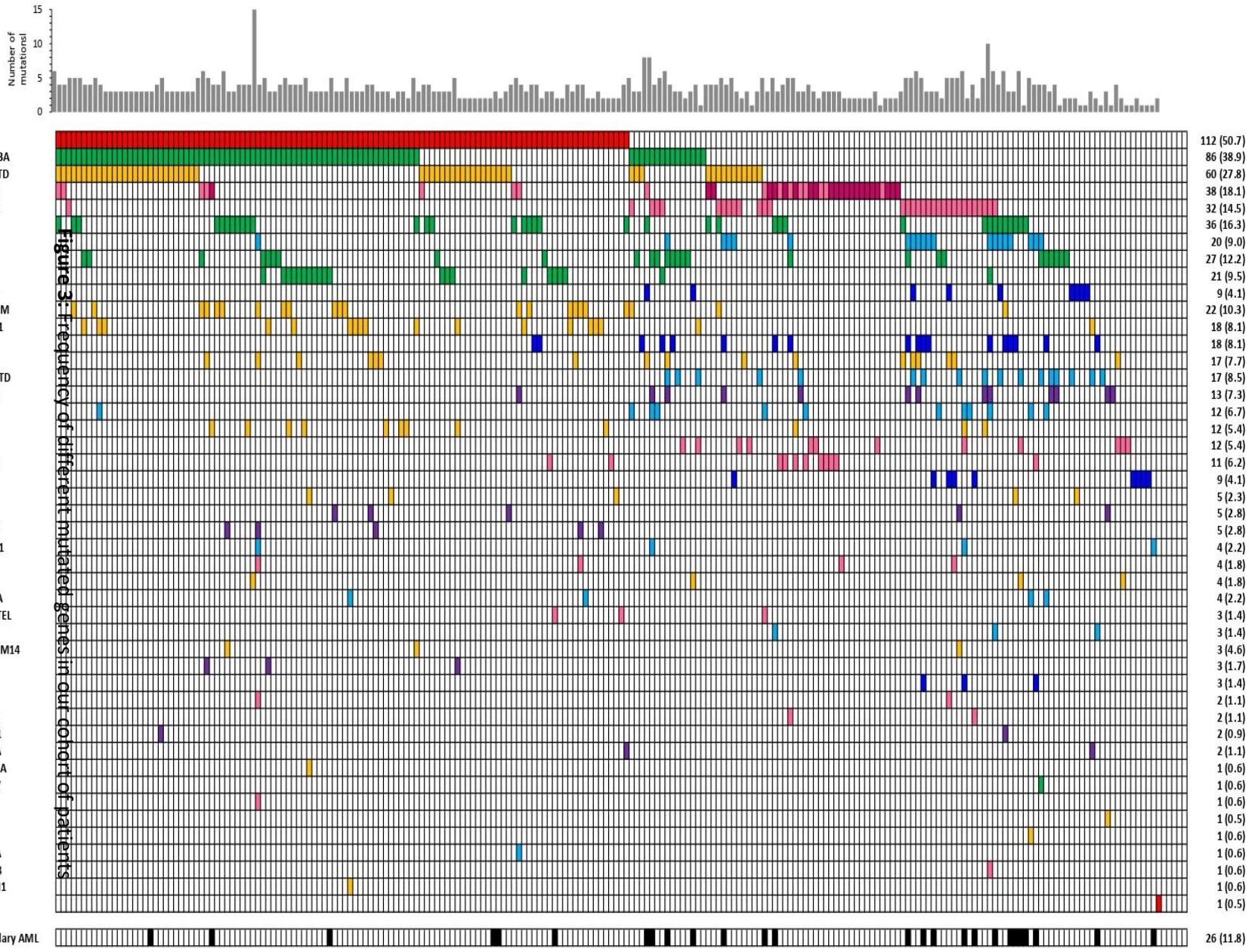


Figure 3: Frequency of different mutated genes in our cohort of patients

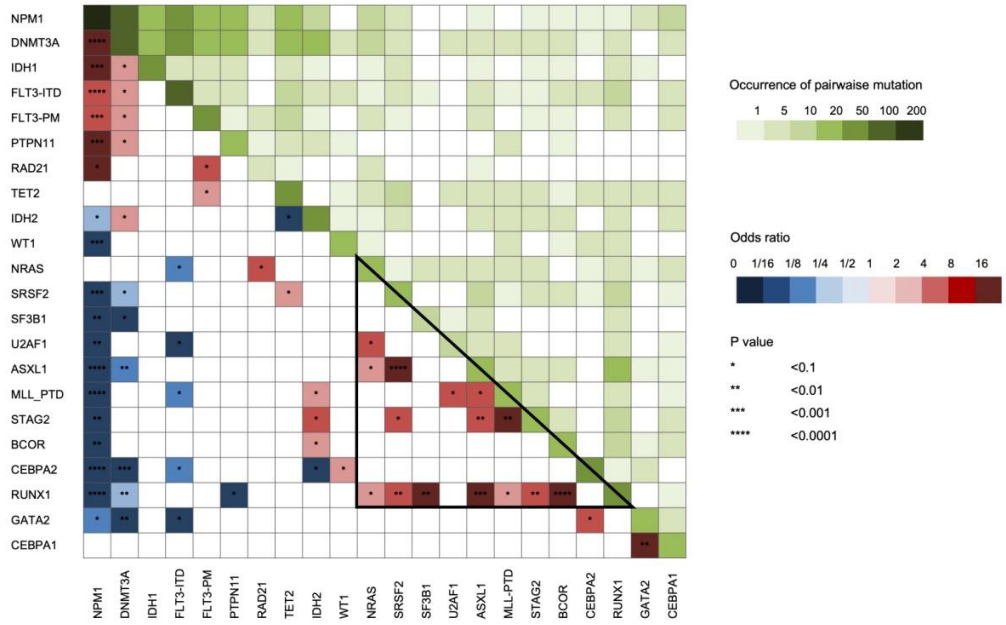


Figure 4: Pairwaise association among gene mutations. The odds ratio of the association is color coded: blue colors indicate a negative association while red colors indicate a positive association. In addition, differential green intensity represent a different co-occurance of mutations in terms of number of patients.

As expected, the most frequently mutated gene in our cohort of patients was *NPM1*, followed by *DNMT3A* and *FLT3*. We noticed that *DNMT3A*, *FLT3*, *IDH1*, *PTPN11*, and *RAD21* mutations were more common in the *NPM1* mutated patients ($p < 0.05$). In particular, *IDH1* R132H mutation was strictly associated with *NPM1* mutation and mutually exclusive with *RUNX1* and *ASXL1* while the R132C was not [13]. Alterations involving the *IDH2* gene in specific amino-acids showed a different behavior regarding co-occurrence with other genes lesions. Particularly, *IDH2* R140

mutation was associated with the presence of *NPM1* alteration and rarely with *RUNX1* mutations, while the amino-acid changes involving R172 presented the opposite combinations [14]. As expected, *RUNX1* mutations often co-occurred with alterations in *ASXL1*, *BCOR*, *SF3B1*, *SRSF2*, *STAG2*, *NRAS*, and *KMT2A*-PTD [15], and within this latter group of genes, pathologic variants were also frequently present in combination (Figure 4). *BCOR* mutations were virtually mutually exclusive with *NPM1* mutations while associated with *RUNX1* alterations [16]. Lastly, *TP53* mutations were revealed only in one NK-AML patient as solely identified genetic aberration (Figure 3). Interestingly, this patient harbored two point mutations probably affecting two different alleles, as commonly described for tumor suppressor genes.

2.3.2. Impact of Clinical and Molecular Profiling on CR Achievement

By univariate analysis, (**Table 2**) age, Eastern Cooperative Oncology Group (ECOG) performance status (PS), de novo AML nature and gene mutation profile at diagnosis had an impact on CR achievement.

	CR		**OS**		**DFS**	
	HR	**P**	**HR**	**P**	**HR**	**P**
HDS	0.94 (0.44-2.03)	0.8731	0.86 (0.59-1.26)	0.4318	0.82 (0.54-1.23)	0.3276
HSCT	-	-	0.31 (0.18-0.51)	0.0000	0.29 (0.17-0.48)	<.0001
Age >60	0.21 (0.09-0.45)	0.0001	2.67 (1.81-3.95)	0.0000	1.92 (1.22-3.02)	0.0047
Sex male	1.67 (0.77-3.79)	0.2019	0.95 (0.65-1.39)	0.7982	1.06 (0.7-1.58)	0.7901

De novo	2.62 (0.94-6.69)	0.0503	0.73 (0.42-1.27)	0.2665	0.76 (0.41-1.43)	0.3975
ECOG PS 2-3	0.25 (0.09-0.73)	0.0076	2.24 (1.25-4.01)	0.0065	1.06 (0.46-2.43)	0.886
WBC count> 50	0.74 (0.34-1.7)	0.4621	1.61 (1.09-2.39)	0.0179	1.37 (0.89-2.12)	0.1533
NPM1	1.76 (0.82-3.92)	0.1541	0.71 (0.48-1.04)	0.0780	0.76 (0.51-1.14)	0.1864
VAF <=40	2.18 (0.87-6.27)	0.1162	0.67 (0.42-1.05)	0.0785	0.89 (0.57-1.39)	0.6075
VAF >40	1.3 (0.5-3.81)	0.6031	0.8 (0.48-1.33)	0.3924	0.59 (0.33-1.08)	0.0866
***FLT3_*ITD**	0.79 (0.34-1.93)	0.5811	2.23 (1.5-3.32)	0.0001	2.18 (1.43-3.33)	0.0003
***FLT3*-ITD low**	0.95 (0.29-4.29)	0.9380	1.67 (0.9-3.08)	0.1032	1.55 (0.8-3.04)	0.1966
***FLT3*-ITD high**	0.87 (0.34-2.51)	0.7813	2.43 (1.56-3.78)	0.0001	2.6 (1.62-4.18)	0.0001
DNMT3A	1.01 (0.47-2.25)	0.9799	1.25 (0.85-1.83)	0.2606	1.49 (0.99-2.23)	0.0553
TET2	0.23 (0.1-0.54)	0.0006	1.38 (0.85-2.24)	0.1926	0.94 (0.5-1.76)	0.8357
RUNX1	0.42 (0.17-1.08)	0.0590	2.25 (1.43-3.55)	0.0005	1.95 (1.15-3.3)	0.0132
IDH2	0.68 (0.25-2.17)	0.4754	0.77 (0.4-1.47)	0.4247	1.05 (0.56-1.97)	0.8732
CEBPA2	3.37 (0.66-61.69)	0.2450	0.26 (0.1-0.71)	0.0088	0.21 (0.06-0.65)	0.007
FLT3_PM	1.57 (0.42-10.17)	0.5605	0.45 (0.2-1.04)	0.0608	0.38 (0.16-0.94)	0.0371
IDH1	0.98 (0.3-4.36)	0.9714	0.95 (0.51-1.78)	0.8781	0.98 (0.51-1.89)	0.9575
ASXL1	0.25 (0.09-0.73)	0.0076	1.54 (0.86-2.76)	0.1434	1.3 (0.63-2.68)	0.4827
CEBPA1	2.32 (0.44-42.85)	0.4243	0.56 (0.23-1.39)	0.2133	0.63 (0.25-1.55)	0.3141
PTPN11	2.95 (0.57-54.09)	0.3022	0.46 (0.19-1.13)	0.0908	0.58 (0.26-1.33)	0.2018
SRSF2	0.12 (0.04-0.34)	0.0001	1.43 (0.77-2.67)	0.2596	0.8 (0.29-2.17)	0.6553
NRAS	0.74 (0.22-3.37)	0.6557	1.46 (0.78-2.72)	0.2387	1.31 (0.64-2.71)	0.4607
KTM2A-PTD	2.42 (0.46-44.57)	0.4027	1.34 (0.67-2.67)	0.4037	1.44 (0.72-2.88)	0.298

STAG2	0.35 (0.1-1.36)	0.0990	1.33 (0.61-2.88)	0.4712	1.3 (0.52-3.23)	0.5691
BCOR	1.95 (0.36-36.43)	0.5310	1.58 (0.73-3.44)	0.2456	1.52 (0.66-3.51)	0.3258
KRAS	1.84 (0.34-34.31)	0.5648	0.81 (0.33-2)	0.6546	1.1 (0.48-2.52)	0.8178
WT1	1.84 (0.34-34.31)	0.5648	0.8 (0.32-1.95)	0.6185	0.75 (0.28-2.04)	0.5736
GATA2	>99.99 (0-NA)	0.9894	0 (0-Inf)	0.9953	0 (0-Inf)	0.9954
SF3B1	1.32 (0.23-24.91)	0.7976	1.42 (0.66-3.06)	0.3689	1.8 (0.83-3.9)	0.1348
U2AF1	1.32 (0.23-24.91)	0.7976	2.69 (1.3-5.55)	0.0075	3.57 (1.64-7.74)	0.0013
***FLT3*-ITDwt, NPM1 wt**	0.58 (0.26-1.28)	0.1744	1.12 (0.76-1.65)	0.5682	1.08 (0.71-1.63)	0.726
***FLT3*-ITD low ratio, *NPM1* wt**	0.81 (0.12-15.82)	0.8505	1.55 (0.57-4.23)	0.388	1.3 (0.41-4.1)	0.6572
***FLT3*-ITD high ratio, *NPM1* wt**	>99.99 (0-NA)	0.9861	2.09 (0.97-4.49)	0.0602	2.47 (1.14-5.34)	0.0214
***FLT3*-ITDwt, *NPM*1 +**	3.27 (1.21-11.42)	0.0336	0.35 (0.21-0.59)	0.0001	0.44 (0.27-0.71)	0.0009
***FLT3*-ITD low ratio, NPM1 +**	1.06 (0.28-7.03)	0.9361	1.27 (0.62-2.62)	0.5089	1.25 (0.58-2.7)	0.5683
***FLT3*-ITD high ratio, *NPM1* +**	0.63 (0.25-1.83)	0.3602	2.15 (1.33-3.47)	0.0017	2.21 (1.31-3.75)	0.0031

Table 2: Univariate analysis on patients outcome

In particular, achievement of CR was negatively affected by the presence of molecular alterations in *TET2*, *ASXL1* and *SRSF2* genes. On the contrary, the group of patients characterized by the presence of an *NPM1* gene mutation in the absence of *FLT3*-ITD showed a significantly higher probability to achieve CR (Table 2). The presence of a double mutation in *CEBPA* gene was associated with a favorable hazard ratio (HR) for CR achievement. By multivariate analysis, the negative effect of the presence of an altered *SRSF2* gene on CR achievement was confirmed (Table 3).

	CR		OS		DFS	
	HR	P	HR	P	HR	P
HSCT	-	-	0.34 (0.19-0.60)	0.0002	0.34 (0.19-0.60)	<.0001
Age >60	0.43 (0.15-1.22)	0.1049	1.37 (0.78-2.40)	0.2661	0.89 (0.51-1.55)	0.6864
De novo	2.17 (0.55-7.76)	0.2460	-	-	-	-
ECOG PS 2-3	0.26 (0.07-1)	0.0398	1.09 (0.42-2.85)	0.8559	-	-
WBC count> 50	-	-	1.20 (0.67-2.14)	0.5456	1.07 (0.63-1.82)	0.8023
NPM1	1.06 (0.33-3.3)	0.9239	0.58 (0.32-1.06)	0.0761	0.49 (0.28-0.88)	0.0163
FLT3_ITD	-	-	2.76 (1.56-4.91)	0.0005	2.81 (1.66-4.73)	0.0001
DNMT3A	-	-	-	-	1.62 (0.95-2.77)	0.0772
TET2	0.4 (0.13-1.24)	0.1048	0.74 (0.33-1.66)	0.4715	-	-
RUNX1	0.54 (0.15-2.03)	0.3501	1.25 (0.60-2.61)	0.5567	0.89 (0.43-1.87)	0.7638
CEBPA2	-	-	0.20 (0.06-0.68)	0.0097	0.17 (0.05-0.57)	0.0040
FLT3_PM	-	-	0.65 (0.23-1.89)	0.4325	0.65 (0.25-1.67)	0.3731
ASXL1	0.87 (0.19-4.63)	0.8628	0.42 (0.16-1.08)	0.0713	-	-
PTPN11	-	-	0.60 (0.21-1.73)	0.3450	-	-
SRSF2	0.24 (0.06-0.95)	0.0376	-	-	-	-
STAG2	0.97 (0.18-6.65)	0.9715	-	-	-	-
SF3B1	-	-	-	-	1.02 (0.42-2.45)	0.9663
U2AF1	-	-	4.19 (1.72-10.23)	0.0016	5.54 (2.25-13.66)	0.0002

Table 3: Multivariable analysis for patients characteristics, treatments and mutations in the complete cohort

The probability to reach CR was not different according to the treatment allocation when a forest plot analysis was applied to each mutation (Figure S1).

2.3.3 Impact of Clinical and Molecular Characteristics on Survival

Survival analysis showed that age, ECOG PS and white blood counts influenced the clinical outcome of NK-AML (Table 2). Mutations of *FLT3 (FLT3-ITD)*, *RUNX1*, and *U2AF1* were associated with a worse OS and DFS ($p < 0.05$) while double alterations involving *CEBPA* gene proved to have a favorable impact on clinical outcome, both in terms of OS and DFS ($p < 0.05$). Patients with *NPM1* gene mutations but negative for *FLT3-ITD* had a better OS and DFS ($p = 0.0001$ and 0.0009, respectively) (Table 2 and Figure S2). This survival advantage was particularly evident in patients randomized to high-dose chemotherapy during the induction phase (Figure S3). Conversely, the presence of *NPM1* gene mutations did not improve the clinical outcome of patients also bearing *FLT3-ITD* alteration. A gradient effect on survival was documented when *FLT3-ITD* positive patients were classified according to ELN guidelines 2017 as low-AR-*FLT3*-ITD (allelic ratio, AR < 0.5) or high-AR (AR ≥ 0.5) (Table 2, Figure 5).

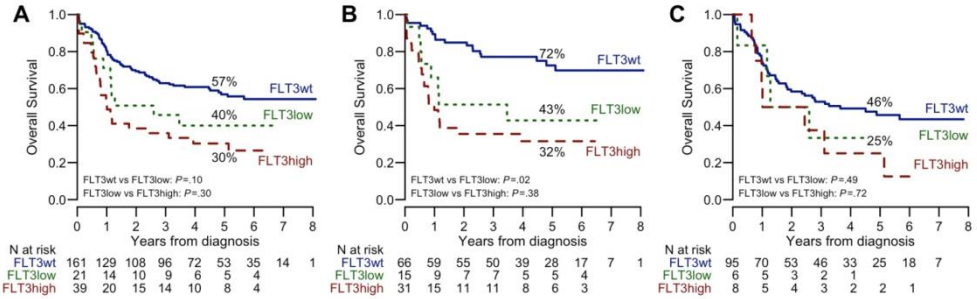


Figure 5: Kaplan-Meier curves of Overall Survival (OS), according to *FLT3*-ITD ratio. (A) All patients; (B) *NPM1* positive patients; (C) *NPM1* wild-type patients. 5-year OS estimates and P values are reported.

We also verified if the allelic burden calculated for *NPM1* mutations (variant allelic fraction, VAF ≤ 0.4 or > 0.4) could have an impact on outcome as recently reported [17] but we did not observe any correlation between *NPM1* VAF and clinical outcome in our cohort of patients. By multivariate analysis (Table 3), the positive effect on survival of an aberrant *NPM1* and a double mutated *CEBPA* was confirmed. In addition, the negative effect on survival related to *FLT3*-ITD as well as mutations involving *U2AF1* gene remained statistically significant also by multivariate analysis.

The univariate analysis showed that the presence of *FLT3*-ITD abolished the prognostic impact of any other identified mutation. By contrast, in patients with no *NPM1* or *FLT3-ITD* mutations, the presence of *DNMT3A*, *TET2*, *RUNX1*, *NRAS,* and *U2AF1* negatively affected survival (Table 4).

	CR n=90		**OS n=90**		**DFS n=75**	
	HR	**P**	**HR**	**P**	**HR**	**P**
HDS	0.51 (0.15-1.59)	0.2622	1.29 (0.72-2.32)	0.3877	1.05 (0.56-1.98)	0.8837
HSCT	-	-	0.43 (0.21-0.89)	0.0229	0.42 (0.2-0.88)	0.0225
Age >60	0.51 (0.16-1.69)	0.2524	2.28 (1.26-4.15)	0.0068	2.63 (1.35-5.11)	0.0043
Sex male	2.38 (0.78-7.77)	0.1336	0.63 (0.35-1.13)	0.1194	0.86 (0.45-1.64)	0.6537
De novo	2.38 (0.65-7.98)	0.1657	0.63 (0.33-1.22)	0.1729	0.58 (0.27-1.23)	0.1569
ECOG PS 2-3	1 (0.15-19.93)	1.0000	2.66 (0.95-7.47)	0.0635	2 (0.61-6.53)	0.2504
WBC count> 50	1.22 (0.19-23.93)	0.8605	0.54 (0.13-2.24)	0.3955	0.28 (0.04-2.03)	0.2066

DNMT3A	0.48 (0.12-2.41)	0.3219	2.77 (1.32-5.8)	0.0068	3.83 (1.67-8.76)	0.0015
TET2	0.21 (0.05-0.81)	0.0195	2.26 (1.09-4.71)	0.0286	2.33 (0.9-5.99)	0.0797
RUNX1	0.41 (0.13-1.37)	0.1323	2.36 (1.28-4.35)	0.0060	1.96 (0.97-3.96)	0.0608
IDH2	0.52 (0.15-2.13)	0.3297	1.03 (0.46-2.3)	0.9439	1.56 (0.68-3.54)	0.2909
CEBPA2	4.5 (0.81-84.34)	0.1598	0.16 (0.05-0.53)	0.0026	0.12 (0.03-0.5)	0.0035
ASXL1	0.27 (0.08-1.03)	0.0466	1.26 (0.59-2.7)	0.5564	1.12 (0.44-2.86)	0.8194
CEBPA1	>99.99 (0-NA)	0.9934	0.45 (0.14-1.45)	0.1801	0.72 (0.25-2.03)	0.5315
SRSF2	0.08 (0.02-0.31)	0.0003	1.33 (0.59-2.98)	0.4889	0.7 (0.17-2.93)	0.6305
NRAS	0.57 (0.11-4.14)	0.5123	2.88 (1.28-6.48)	0.0105	2.9 (1.13-7.48)	0.0272
KTM2A-PTD	3.5 (0.62-65.89)	0.2437	1.05 (0.49-2.26)	0.8960	1.22 (0.56-2.66)	0.6168
STAG2	0.79 (0.17-5.67)	0.7783	1.16 (0.45-2.98)	0.7603	1.57 (0.6-4.09)	0.3593
BCOR	>99.99 (0-NA)	0.9908	1.31 (0.46-3.71)	0.6128	1.36 (0.48-3.9)	0.5639
KRAS	>99.99 (0-NA)	0.9914	0.61 (0.08-4.44)	0.6276	0.6 (0.08-4.37)	0.6138
WT1	>99.99 (0-NA)	0.9903	0.58 (0.18-1.86)	0.3584	0.6 (0.18-1.96)	0.3983
GATA2	>99.99 (0-NA)	0.9937	0 (0-Inf)	0.9973	0 (0->99.99)	0.9973
SF3B1	1.44 (0.23-28.05)	0.7417	1.4 (0.59-3.3)	0.4479	1.77 (0.74-4.26)	0.2010
U2AF1	1.67 (0.27-32.28)	0.6406	3.03 (1.4-6.58)	0.0049	3.89 (1.69-8.93)	0.0014

Table 4: Univariate analysis for patients characteristics, treatments and mutations in patients lacking both FLT3-ITD and NPM1 alterations (n=90 and n=75 achieving CR)

In this subgroup, the unfavorable prognostic effect of *U2AF1* mutations on survival remained significant also by multivariate analysis. The presence of a *RUNX1* mutation was associated with an unfavorable, despite not statistically significant, HR for survival (Table 5).

	CR		OS		DFS	
	HR (95% CI)	**P**	**HR (95% CI)**	**P**	**HR (95% CI)**	**P**
HSCT	-	-	0.42 (0.16-1.09)	0.0744	0.24 (0.09-0.62)	0.0032
Age >60	-	-	1.02 (0.43-2.41)	0.9624	1.12 (0.51-2.45)	0.7778
Sex male	2.57 (0.66-11.53)	0.1846	0.77 (0.33-1.78)	0.5462	-	-
De novo	0.65 (0.11-2.96)	0.5985	0.88 (0.36-2.16)	0.7753	1.29 (0.50-3.33)	0.5997
ECOG PS 2-3	-	-	2.24 (0.54-9.31)	0.2681	-	-
DNMT3A	-	-	2.58 (0.80-8.28)	0.1105	3.57 (1.07-11.89)	0.0383
TET2	0.15 (0.02-0.94)	0.0387	2.32 (0.70-7.63)	0.1670	1.93 (0.62-6.03)	0.2600
RUNX1	0.44 (0.1-1.99)	0.2747	2.20 (0.93-5.24)	0.0741	1.93 (0.83-4.50)	0.1277
CEBPA2	3.93 (0.47-98.06)	0.2806	0.20 (0.04-0.92)	0.0387	0.13 (0.03-0.58)	0.0070
ASXL1	1.38 (0.24-11.14)	0.7367	-	-	-	-
NRAS	-	-	1.21 (0.38-3.87)	0.7457	1.05 (0.34-3.29)	0.9284
SRSF2	0.08 (0.01-0.5)	0.0093	-	-	-	-
U2AF1	-	-	3.39 (1.16-9.92)	0.0260	3.81 (1.35-10.78)	0.0117

** CEBPA2* indicates the presence of a double mutation.

Table 5: Multivariate analysis for patients characteristics, treatments and mutations in *FLT3* wt and *NPM1* wt patients.

2.3.4. Impact of alloHSCT by Molecular Lesions

The 22 FLT3-ITD positive patients who could proceed to alloHSCT had a survival probability similar to FLT3-ITD negative patients. The transplant outcome was not different when comparing high and low-AR-FLT3-ITD

subgroups both in terms of OS (Figure 6) and DFS. The OS of FLT3-ITD positive patients, no matter if NPM1 negative or positive, who did not receive alloHSCT for whatever reason showed quite a poor outcome (Figure 6B, $p < 0.00001$). The limited number of patients precluded the possibility to evaluate the ability of alloHSCT to modify the adverse outcome associated with other molecular alterations.

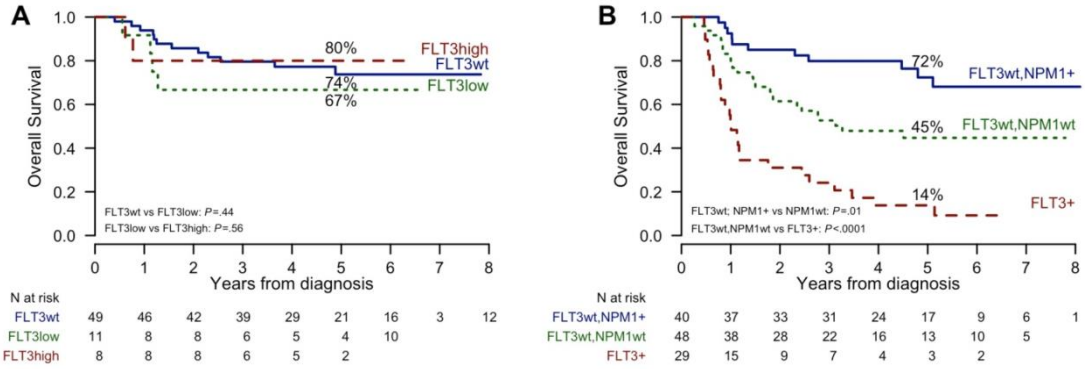


Figure 6: Kaplan-Meier curves of Overall Survival (OS) in different consolidation programs. (**A**) Patients receiving allogeneic stem cell transplantation, according to *FLT3*-ITD ratio; (**B**) Patients receiving other consolidation program, according to *FLT3*-ITD and *NPM1* mutations. 5-year OS estimates and *p* values are reported.

2.3. Discussion

In this study, we provide an accurate molecular characterization of 221 NK-AML patients included in a prospective clinical trial comparing the standard ICE induction chemotherapy to the high-dose regimen. By applying an NGS high throughput solution to sequence myeloid neoplasms related genes, we

were able to identify at least one mutation in the great majority of patients (98%). Frequencies and co-occurrence of mutations are consistent with previous observations [3,14]. Our data confirm that the identification of *CEBPA*, *NPM1* and *FLT3-ITD* mutations, alone or in combination, remains crucial to define patient subgroups with different prognoses. Double mutation in *CEBPA* gene identifies a subgroup of patient characterized by a particularly favorable outcome. On the contrary, *FLT3*-ITD mutations represent the most relevant marker of unfavorable prognosis in this setting, no matter the presence of *NPM1* or other gene mutations. We observed a gradient effect played by *FLT3*-ITD allele burden on survival since, the low-AR-*FLT3*-ITD was still associated to a negative outcome. This correlation was not statistically significant probably due to the sample size of low-AR-FLT3-ITD subgroups (with or without mutant *NPM1*) which is relatively low. This observation is in line with other studies [9,18] and represents an open challenge as to the choice of the post-remission strategies. Within the limit of a modest number of patients so far analyzed, our results suggest that alloHSCT can abolish the adverse effect due to the *FLT3*-ITD mutation. For these reasons, at our institution, alloHSCT remains the preferred post remission option for patients with low-AR-*FLT3*-ITD. The role of innovative *FLT3* inhibitors, either to improve the transplant outcome or to avoid it, will perhaps modify the therapeutic scenario of this AML subgroup [19,20,21]. The *FLT3*-ITD mutation exerts its negative influence also in *NPM1* mutated patients. This observation supports the paradigm of how the presence of co-occurring mutations can modify the effect of a single mutation on the prognosis [22] and demonstrates the importance of refining molecular characterization of AML at disease presentation.

In patients with no mutations of both *FLT3*-ITD and *NPM1*, additional mutations in other leukemia-related genes proved to influence disease evolution. Therefore, the identification of specific mutations in this subgroup is mandatory to predict the clinical outcome and to select the most appropriate treatment approach. We found that molecular lesions in *TET2*, *SRSF2*, and *U2AF1* were associated with negative outcomes. Our data are in line with recent studies showing that *TET2* mutations and older age are independent prognostic factor in AML [23]. The *U2AF1* adverse prognostic impact on survival has been already reported in a limited AML cohort [**24**]. To the best of our knowledge, the data on the impact of *SRSF2* mutations on CR achievement were not previously reported in a cohort of patients with AML.

For the few patients (2%) with no evidence of DNA mutations, sequencing of a wider genome region, including regulatory and intronic sequences, and/or the use of an integrate analysis including other approach as comparative genomic hybridization arrays might identify rarer AML related genetic abnormalities and provide useful information for clinical decision making [25].

2.4. Patients and Methods

Out of 574 newly diagnosed AML patients enrolled into the NILG-AML 02/06 clinical trial, 270 subjects showed a normal karyotype. Molecular profile was performed on a total of 221 NK-AML with available diagnostic samples. Patients were affected by a de novo AML or by an AML secondary to chemo-radiotherapy or to a myelodysplastic/myeloproliferative syndrome (Table

1). This protocol was a randomized trial comparing ICE (idarubicin-cytarabine-etoposide) with sequential high dose (HD) chemotherapy in untreated patients with the intent to improve the early remission rate and to evaluate the impact on survival [12]. The trial protocol has been approved by the institutional review boards at each of the participating center (Comitato etico della provincia di Bergamo (CE150180), Comitato Etico Area Vasta Centro (CE150071), Comitato Etico città della salute e della scienza (CE150115), Comitato Etico Brianza (CE150179), Comitato Etico Interaziendale A.S.O. SS. Antonio e Biagio e C.Arrigo di Alessandria (CE150105), Comitato Etico di Brescia (CE150186), Comitato etico per la sperimentazione clinica - Comprensorio di Bolzano (CE150099), Comitato Etico Interaziendale Aso S.Croce E Carle (CE150123), CESC della Provincia di Venezia e IRCSS San Camillo(CE150073), Comitato Etico Val Padana (CE150177), Comitato Etico dell'IRCCS San Raffaele (CE150050), Comitato Etico Indipendente Istituto Clinico Humanitas (CE150081), Comitato Etico dell'Insubria (CE150185), Comitato Etico Indipendente della Fondazione IRCCS Istituto Nazionale dei Tumori di Milano (CE150053) and Comitato Etico Milano Area 2 (CE150176)).Informed consents for inclusion in the trial and for genetic analysis were obtained from all patients. Genomic DNA was isolated from mononuclear cells obtained from bone marrow or peripheral blood at diagnosis, containing at least 20% blasts. In the analysis of *FLT3*-ITD and D835 point mutations, *KTM2A-PTD*, *NPM1,* and *CEBPA* alterations were prospectively obtained with standard approaches (PCR analysis, enzymatic digestion, Sanger sequencing). In addition, we estimated the mutant to wild-type allelic ratio (AR) of *FLT3-ITD* using fragment length analysis technique [26]. Subsequently, on the same prospectively collected diagnostic samples,

we obtained a more complete molecular profile by next generation sequencing (NGS) of targeted regions of a wide selection of myeloid neoplasms related genes. Two commercial NGS kits were applied to prepare DNA libraries for sequencing: Trusight Myeloid panel (Illumina, San Diego, CA, USA) and Sophia Myeloid Solution (SOPHiA GENETICS, SA, CH) investigating 54 and 30 gene regions, respectively (Table S1). The libraries were sequenced and demultiplexed on a MiSeq or MiniSeq instruments (Illumina, San Diego, CA). The median coverage was 6373 reads (range 44166–103) with 92% sequenced regions with > 500 and 87% with > 1000 reads. The limit of detection (LOD) for a reliable variant calling was down to 5% variant allele frequency (VAF), as recommended by both the producers. Frameshift and nonsense variants were always considered as relevant mutations. Single nucleotide variants were retained in the absence of description as genetic polymorphism into public databases of human polymorphisms (NCBI dbSNP (http://www.ncbi.nlm.nih.gov/snp; Build 137) and ExAC (http://exac.broadinstitute.org/)). Functional prediction for missense variants was derived from SIFT 1.03 (http://sift.jcvi.org) and PolyPhen2.0 (http://genetics.bwh.harvard.edu/pph2). For alterations of splicing sites and splicing related regions, we used the Human Splicing tool (Human Splicing Finder) to predict the effect on the splicing process. Finally, the description of other cancer specimens in terms of the identified mutations was checked against COSMIC database (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic).

The clinical endpoints of the study were defined according to standard criteria [27]. Overall survival (OS) was defined as the probability of survival

irrespective of disease state at any point in time from diagnosis. Patients alive at their last follow-up were censored. Disease free survival (DFS) was measured from the time of first CR until relapse or death. Baseline continuous characteristics were presented as median with range and compared using the Mann–Whitney U test. Categorical variables were reported with absolute and percentage frequencies and compared with Chi-squared test or Fisher's exact test. OS and DFS were estimated by the Kaplan–Meier method and any differences were evaluated with a log-rank test. Cox models were used to estimate hazard ratios with 95% confidence intervals (CI) in univariate and multivariable analysis on survival outcomes. In this context, allogeneic hematologic stem cell transplantation (alloHSCT) was considered as a time-dependent event; Mantel–Byar tests and Simon–Makuch plots were used. In multivariable models, only factors with a *p* value < 0.2 in a corresponding univariate model were included. All reported *p* values are two-sided and a 5% significance level was set. All analyses were performed with R software, version 3.5.0.

2.5. Conclusions

In NK-AML, the accurate and in-depth molecular characterization did not lead to the recognition of a mutational profile associated with a different rate of response following an intensified induction chemotherapy program. No matter the induction chemotherapy, we identified mutations which are associated with different outcomes and which help to select the most appropriate consolidation strategies, namely alloHSCT. Finally, the identification of mutations that represent a potential treatment target for

new drugs is now mandatory for offering patients new chemotherapy free therapeutic options.

2.6 Supplementary Materials

Figure S1: Kaplan-Meier curves of Overall Survival (OS) in different induction treatments, according to *FLT3*-ITD and *NPM1* mutations. 3-year OS estimates and global P values are reported.

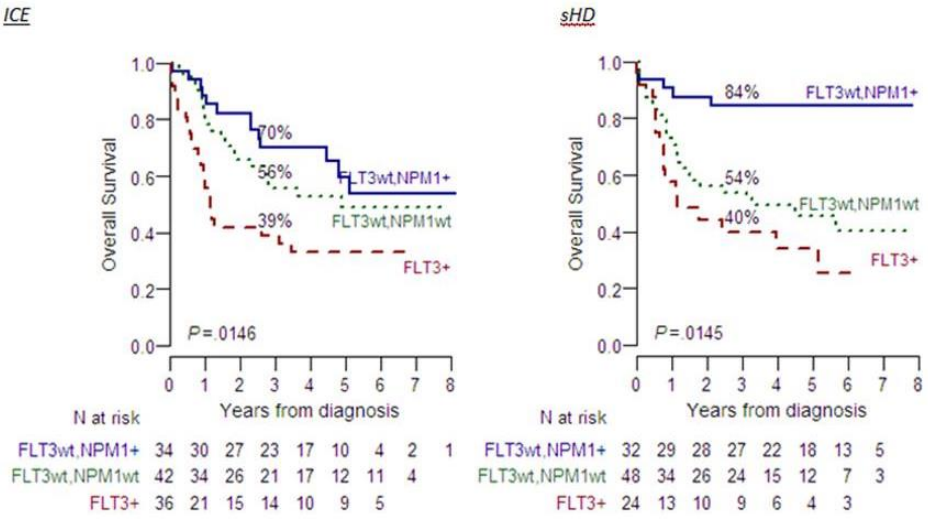


Figure S2: Forest plot of induction treatment. Effects of treatments on CR, according to the gene molecular alteration detected in our cohort of patients.

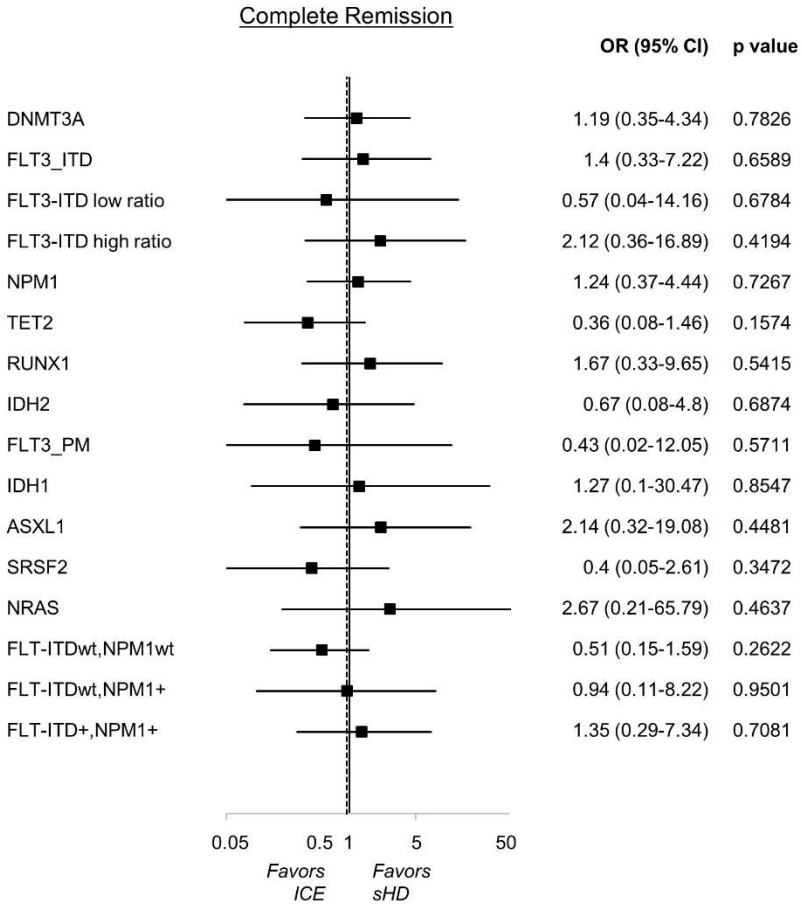
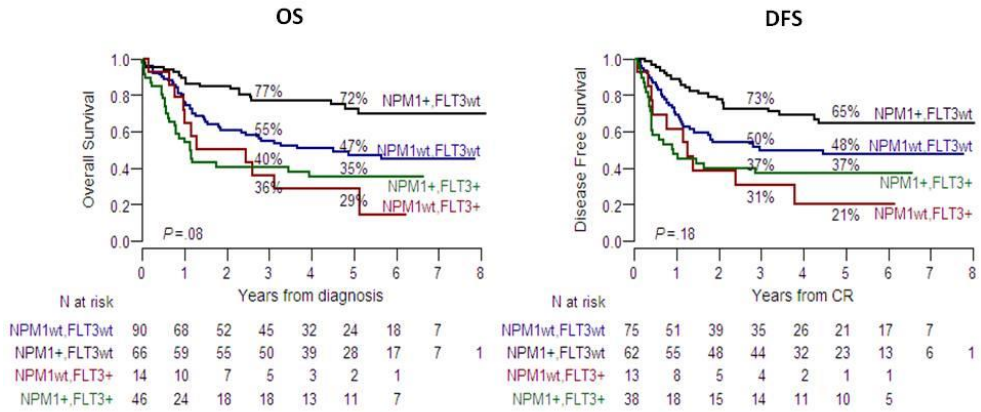


Figure S3: Kaplan-Meier curves of Overall Survival and Disease-free Survival according to *FLT3*-ITD and *NPM1* mutations. 3-year and 5-year estimates and global P values are reported.



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CHAPTER 3

IDENTIFICATION OF A CHROMATIN-SPLICEOSOME MUTATIONAL SIGNATURE TO DEFINE SECONDARY ACUTE MYELOID LEUKEMIA

Identification of a Chromatin-Spliceosome Mutational Signature to Define Secondary Acute Myeloid Leukemia

Chiara Caprioli*[1], Federico Lussana*[1], Silvia Salmoiraghi[1], Roberta Cavagna[1], Ksenija Buklijas[1], Lara Elidi[1], Pamela Zanghì[1], Anna Michelato[1], Federica Delaini[1], Elena Oldani[1], Tamara Intermesoli[1], Anna Grassi[1], Giacomo Gianfaldoni[2], Mannelli Francesco[2], Dario Ferrero[3], Ernesta Audisio[3], Elisabetta Terruzzi[4], Lorella De Paoli[5], Giuseppe Rossi[6], Erika Borlenghi[6], Irene Cavattoni[7], Monica Tajana[8], Anna Maria Scattolin[9], Daniele Mattei[10], Paolo Corradini[11], Leonardo Campiotti[12], Fabio Ciceri[13], Massimo Bernardi[13], Elisabetta Todisco[14], Agostino Cortelezzi[15], Brunangelo Falini[16], Chiara Pavoni[1], Renato Bassan[9], Orietta Spinelli[1], and Alessandro Rambaldi[1,17]

1 Azienda Socio-Sanitaria Territoriale (ASST) Ospedale Papa Giovanni XXIII, Bergamo, Italy; 2 Azienda Ospedaliera Universitaria Careggi (AOU), Firenze, Italy; 3 AOU Città della Salute e della Scienza, Torino, Italy

4 Azienda Ospedaliera San Gerardo, Monza, Italy; 5 Azienda Ospedaliera SS. Antonio e Biagio e Cesare Arrigo, Alessandria, Italy; 6 ASST Spedali Civili, Brescia, Italy; 7 Ospedale San Maurizio, Bolzano, Italy; 8 ASST Ospedale di

Cremona, Cremona, Italy; 9 Ospedale dell'Angelo e SS. Giovanni e Paolo, Venezia Mestre, Italy; 10 Azienda Ospedaliera S. Croce e Carle, Cuneo, Italy; 11 Fondazione Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Istituto Nazionale dei Tumori, Milano, Italy; 12 Università dell'Insubria, Varese, Italy; 13 IRCCS Ospedale San Raffaele, Milano, Italy; 14 IRCCS Istituto Clinico Humanitas di Rozzano, Rozzano, Italy; 15 Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milano, Italy; 16 Università di Perugia, Perugia, Italy; 17 Department of Oncology and Hematology, Università degli Studi di Milano, Milano, Italy

*These authors contributed equally to this work.

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3.1. Abstract

Secondary acute myeloid leukemia (sAML) after myelodysplastic (MDS) or myeloproliferative neoplasms (MPN) shows poorer outcomes compared to *de novo* AML. This high-risk category is currently identified by clinical history or specific morphological and cytogenetic abnormalities. However, in the absence of these features, uncertainties remain to identify the secondary nature of some cases otherwise defined as *de novo* AML. Aiming to implement the current definition of sAML, we analyzed a prospective cohort of 413 newly diagnosed AML patients focusing on a chromatin-spliceosome mutational signature. Chromatin-spliceosome mutations were identified in 17.6% of clinically defined *de novo* cases, which showed clinical characteristics closer to sAML (older age, lower white blood cell counts and higher rate of multilineage dysplasia). Clinical outcomes in this group were adverse and comparable to those of patients with sAML (overall survival 30% and 17% respectively vs 61% of *de novo;* disease free survival 26% and 22% respectively vs 54% of *de novo*; $P<0.001$ for both comparisons) and independently confirmed by multivariable analysis. Allogeneic hematopoietic stem cell transplantation (alloHSCT) performed in first complete remission (CR) improved outcomes in both sAML and CS-AML

patients. These data support a molecular definition of sAML, with potential implications for optimized treatment choice.

3.2. Introduction

According to the current WHO classification (1), sAML is defined either by a previous clinical history of hematological disease, the morphological detection of multilineage dysplasia or specific cytogenetic characteristics; the two latter criteria are additive to clinical history or may be themselves sufficient to diagnose sAML, even in the absence of a known antecedent MDS or MPN phase (1,2). However, in clinical practice some uncertainty remains regarding the correct classification of potentially high-risk sAML cases, especially when antecedent history is not thoroughly documented or does not fully satisfy the diagnostic criteria of MDS. Moreover, morphological examination to assess blast counts and multilineage dysplasia shows inter-observer variability that may impair diagnostic reproducibility (3). Finally, cytogenetic analysis, which usually takes a long turnaround time of 5-10 days, may not be always informative because of technical failure or normal result. These features eventually translate into under-recognition of sAML patients and lead to inadequate clinical management, since this high-risk population deems intensive treatment strategies comprising the administration of innovative agents or allocation to clinical trials, potentially able to improve the rates of CR (4–7), followed by a rapid consolidation with

alloHSCT (7,8). Therefore, more accurate diagnostic approaches are warranted. In this regard, studies focusing on the molecular landscape of sAML and preceding conditions have suggested the possibility of defining distinct subtypes of AML based on their mutational profiles. Mutations in genes involved in chromatin regulation (*ASXL1, EZH2, BCOR, STAG2*) and RNA splicing (*SRSF2, SF3B1, U2AF1, ZRSR2*) have shown high specificity for sAML after MDS (9); these mutations,called secondary-type mutations, occur early in leukemogenesis (likely representing the expansion of clones acquired during previous MDS) (10,11) and often persist in clinical remission, presenting as constitutively chemo-resistant. Although less characterized, sAML cases progressing after MPN display similar features (12,13). However, a closely related mutational signature can be identified also in some *de novo* AML cases (14,15). In the seminal study conducted by Papaemmanuil et al. (14) on a large cohort of AML patients, overlapping mutations in genes regulating RNA splicing (*SRSF2, SF3B1, U2AF1,* and *ZRSR2*), chromatin (*ASXL1, STAG2, BCOR, KMT2A*-PTD, *EZH2*, and *PHF6*), or transcription (*RUNX1*) constituted a homogeneous genomic class, called the chromatin–spliceosome group. Strikingly, 91% of patients in this group had a *de novo* AML by clinical definition; the molecular signature was associated with older

age, lower white blood cell and blast counts, lower rates of response to induction chemotherapy and higher relapse rates. While these results warrant prospective validation, it can be hypothesized that the presence of these mutations represent the trace of a previous, unrecognized MDS or MPN phase. Nevertheless, a formal comparison between *de novo* AML patients carrying chromatin-spliceosome mutations and sAML patients defined by standard criteria is currently lacking.

In this study, we investigated the validity of a molecular definition of sAML by re-assessing patients' diagnosis according to the presence of chromatin-spliceosome mutations in a large cohort of newly diagnosed AML patients enrolled into a prospective trial (NILG AML 02/06) [ClinicalTrials.gov Identifier: NCT00495287] (16). Here, we report the characteristics and outcomes of initially defined *de novo* AML patients carrying chromatin-spliceosome mutations as compared with other clinically defined *de novo* AML patients without chromatin-spliceosome mutations and patients with sAML defined by standard WHO criteria.

3.3. Materials and Methods

3.3.1. Patients and treatment

The NILG-AML 02/06 trial (16) enrolled 574 adult patients with newly diagnosed AML [≥20% bone marrow blasts (BM), excluding acute promyelocytic leukemia] or high-risk MDS (10-19% BM blasts) across 17 Italian centers between 2007 and 2012. AML diagnosis was locally confirmed following the standard criteria adopted for this trial and patient enrollment required central review of diagnostic BM slides and trephine biopsy. All participants were randomized to receive induction chemotherapy, either a conventional regimen including idarubicin, cytarabine and etoposide (ICE) or a high-dose regimen with sequential administration of cytarabine and idarubicin (sHD). Patients not responding to first induction cycle underwent an intensified re-induction with high-dose cytarabine. Post-induction treatment was based on a study-specific risk stratification and included consolidation courses with high-dose cytarabine, autologous stem cell transplant or alloHSCT for high-risk patients (full details have been previously reported) (15). The trial protocol was in accordance with the Declaration of Helsinki and has been approved by the institutional review boards at each of the participating centers. All patients provided written informed consent for inclusion into the clinical trial and for genetic studies.

3.3.2. Cytogenetic and molecular analyses

Conventional karyotype was obtained at diagnosis at each of the participating centers; informative results were available for 413 patients.

Molecular analyses were performed centrally at "Paolo Belli" Lab (Department of Hematology, Bergamo Hospital) on samples prospectively collected at diagnosis before any treatment administration. Specifically, analyses were performed on mononuclear cells obtained by Ficoll-gradient centrifugation from peripheral blood and/or BM containing at least 20% blasts. *NPM1*, *FLT3*-ITD, *FLT3* point mutations, *RUNX1-RUNX1T1*, *CBFb-MYH11*, biallelic *CEBPa* and *KMT2A*-PTD mutations were tested on the whole cohort using PCR, Sanger sequencing and/or fragment analysis. Targeted NGS was performed on 196 patients with normal karyotype using two commercial kits: an amplicon-based method (Trusight Myeloid panel by Illumina, San Diego, California, USA; n=161) amplifying 54 gene regions and a capture-based method (Sophia Myeloid Solution by Sophia Genetics SA, Saint Sulpice, Switzerland; n=35) selecting 30 gene regions for library preparation. Libraries were sequenced and demultiplexed on a MiSeq or MiniSeq instrument (Illumina). The detection limit/sensitivity for identified variants was set to 5% variant allele frequency. Frameshift and nonsense

variants were always considered as relevant mutations. Single nucleotide variants were retained in the absence of description as genetic polymorphism into public databases of human polymorphisms (NCBI dbSNP (http://www.ncbi.nlm.nih.gov/snp; Build 137) and Exac (http://exac.broadinstitute.org/). Functional interpretation for missense variants was performed using SIFT 1.03 (http://sift.jcvi.org) and PolyPhen2.0 (http://genetics.bwh.harvard.edu/pph2). For alterations of splicing sites and splicing related regions, we used the Human Splicing tool (http://www.umd.be/HSF3) to predict alterations in the splicing process. Indeed, the description of the identified mutations in literature was checked in COSMIC database (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic).

The chromatin-spliceosome mutational signature included mutations of *ASXL1, STAG2, BCOR, KMT2A*-PTD*, EZH2, PHF6, SRSF2, SF3B1, U2AF1, ZRS2* and *RUNX1,* according to Papaemmanuil et al. (14).

3.3.3. Definition of AML categories

To perform comparative analyses, we defined the following AML categories among patients enrolled into the trial: 1) chromatin-spliceosome (CS)-AML: clinically defined *de novo* AML patients carrying at least one chromatin-spliceosome mutation among *ASXL1, STAG2, BCOR, KMT2A*-PTD*, EZH2, PHF6, SRSF2, SF3B1, U2AF1, ZRS2* and *RUNX1*, excluding patients with concurrent WHO-recurrent abnormalities (1); 2) sAML: patients with a previous documented clinical history of MDS, MDS/MPN or MPN and/or cytogenetic WHO criteria of AML with MDS-related changes (1); 3) *de novo* AML: none of the above.

We discarded MDS patients, therapy-related AML patients and patients not provided with a cytogenetic and/or molecular characterization. To exclude possible ambiguities in interpretation, morphological WHO criteria of AML with MDS-related changes were not considered in the definition of sAML.

3.3.4. Study endpoints and statistical methods

The clinical endpoints of the study were defined according to standard criteria (17). CR was defined as achievement of less than 5% BM myeloblasts after 1 or 2 induction cycles (whichever comes first), in the absence of circulating blasts, blasts with Auer rods or extramedullary disease.

Refractory patients were defined as not achieving CR after 2 induction cycles. Early death was defined as death by any cause during or immediately after first induction cycle, with aplastic or indeterminate BM status. Overall survival (OS) was defined as the probability of survival irrespective of disease state at any point in time from diagnosis. Patients alive at their last follow-up were censored. Disease free survival (DFS) was measured from the time of first CR until relapse or death. Relapse was defined by recurrence of more than 5% myeloblasts in the peripheral blood or in the BM and/or by the presence of extramedullary disease.

Baseline continuous characteristics were presented as median with range and compared using the Mann-Whitney U test. Categorical variables were reported with absolute and percentage frequencies and compared with Chi-squared test or Fisher's exact test.

OS and DFS were estimated by the Kaplan-Meier method and any differences between AML categories or consolidation treatment were evaluated with log-rank test. Cox models were used to estimate hazard ratios with 95% confidence intervals (CI) in univariate and multivariable analysis on survival outcomes. In this context, alloHSCT was considered as a time-dependent event; outcome data were estimated by the Mantel-Byar

method and graphically illustrated by Simon-Makuch plots. All reported *P* values are two-sided and a 5% significance level was set. All analyses were performed with R software, version 3.5.0.

3.4. Results

3.4.1. Characteristics of patients

Among 574 adult patients enrolled in the NILG-AML 02/06 trial (16), 413 (72%) with full genetic characterization resulted evaluable and were classified as 55 CS-AML patients, 100 sAML patients (28 defined by clinical history, of which 24 after MDS or MDS/MPN and 4 after MPN, and 72 defined by cytogenetic criteria) and 258 *de novo* AML patients (Figure 1). CS-AML was recognized in 17.6% of otherwise defined *de novo* AML cases and represented a significant proportion of the whole analyzed cohort (13%).

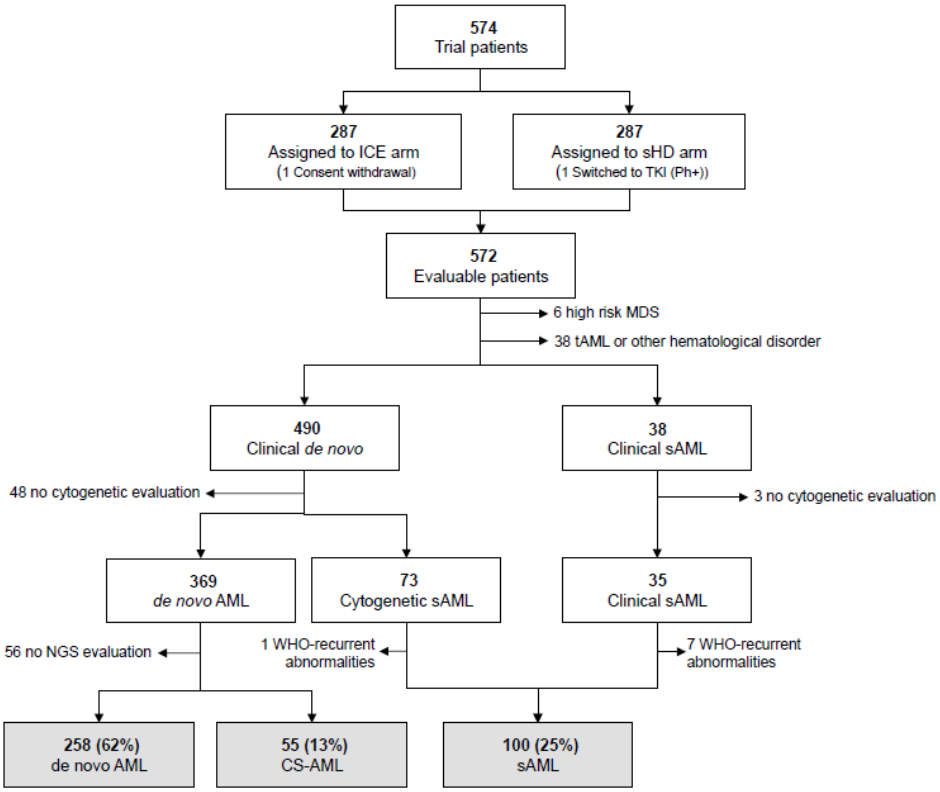


Figure 1. CONSORT diagram illustrating patient selection. ICE, idarubicin, cytarabine, etoposide; sHD, sequential high-dose chemotherapy; TKI, tyrosine-kinase inhibitors; tAML, therapy-related acute myeloid leukemia.

The main clinical characteristics of patients are reported in Table 1. Compared to *de novo* AML, patients with sAML and CS-AML were similarly older (median age 48, 59 and 58 years respectively, $P<0.0001$) and presented at diagnosis with lower white blood cell counts (WBC) ($P<0.0001$), with no significant differences between the two latter categories. A lower BM blast infiltration was reported in sAML as compared with both *de novo*

AML (*P*<0.0001) and CS-AML (*P*=0.02). By morphological analysis, multilineage dysplasia was described at diagnosis in a minor proportion of CS-AML patients (11%), close to that of sAML patients (9%, *P*=0.77) and similarly higher than that of *de novo* AML patients (2%, *P*=0.0051).

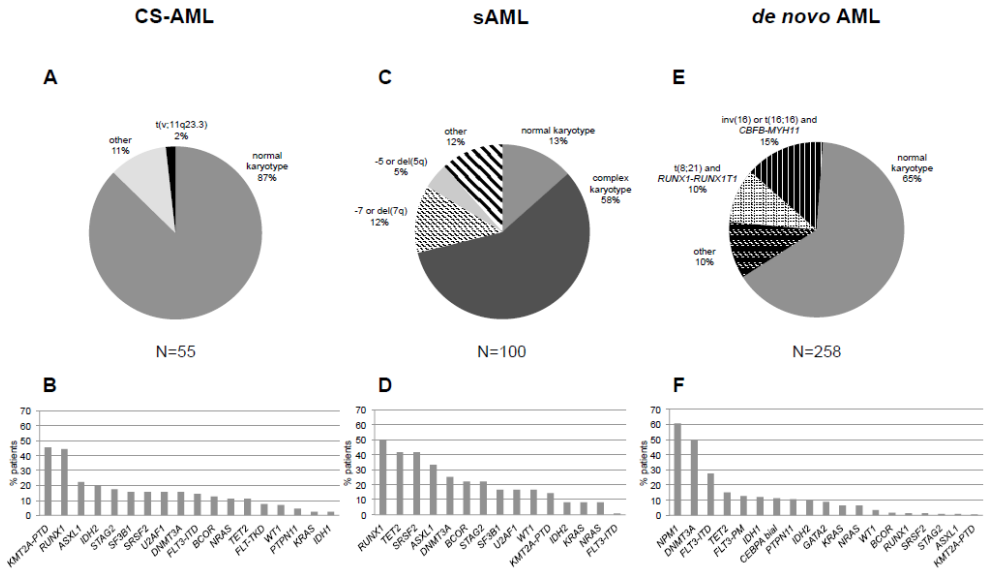


Figure 2. Cytogenetic and molecular characteristics of AML categories. For each AML category, pie charts (top of the figure) depict the distribution of chromosomal abnormalities, while histograms (bottom) show the frequency of individual mutations. (A, B) CS-AML, (C, D) sAML and (E, F) *de novo* AML. The label "other" includes: for the CS-AML category, abnormalities of chromosome 11 [(other than t(v;11q23.3) and del(11q)] and +8; for the sAML category, del(11q), +8, del(12p), t(5q;12p), t(1p;3q), t(3q;5q) and -Y; for the *de novo* AML category, +8, del(9q), +21, monosomy 21, +13, t(8q;11q), inv(3), monosomy X, -Y, del(16q), add(4q), add(6p), t(13p;17p).

The cytogenetic and molecular characteristics of the cohort are summarized in Figure 2. In the CS-AML category (N=55) (Figure 2A-B), the majority of patients (87%) had a normal karyotype; 160 mutations were found in total, with a median of 3 mutations per patient (range 1-6). The most frequently reported mutations of the chromatin-spliceosome signature were in *KMT2A* (*KMT2A*-PTD), *RUNX1* and *ASXL1* genes (respectively 45.5%, 44.4% and 22.2% of evaluable patients), while other mutations accounted for 5-17.5% of cases. While 54.5% of patients in this category presented with a single chromatin-spliceosome mutation, overlap and significant associations between chromatin-spliceosome mutations were observed in 45.5% of cases. Other mutations scored in CS-AML patients included *IDH2, DNMT3A, FLT3*-ITD, *TET2* and *NRAS* (respectively 20%, 15.6%, 14.5% and 11% of evaluable patients), and others (Figure 3 and Figure 4). Among the 100 sAML patients (Figure 2C-D), a huge proportion carried a complex karyotype (58%) and abnormalities of chromosome 7 (12%) or 5 (5%). Nevertheless, 13% of patients in this category had a normal karyotype; 62 mutations were reported in total (median 0, range 0-8), mostly involving chromatin-spliceosome genes. The *de novo* AML category (N=258) (Figure 2E-F) included 25% of patients with a core binding factor

AML, a high prevalence (65%) of patients with normal karyotype and 10% of patients with other non WHO-recurrent cytogenetic abnormalities. A total of 607 mutations were scored (median 2 per patient, range 0-15), the most frequently represented being *NPM1* (60.7% of patients), *DNMT3A* (49.3%) and *FLT3*-ITD (27.5%).

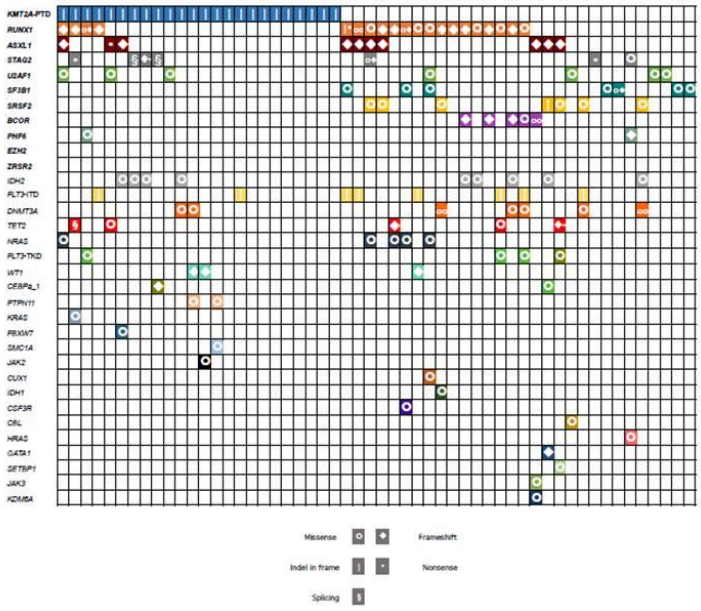


Figure 3. Mutational profile of 55 CS-AML patients. Each column represents an individual CS-AML patient, while each row represents a single gene mutation out of the list at the left. Colored bars indicate the presence of one or more mutations of each gene. Variant types are specified according to the legend at the bottom of the figure.

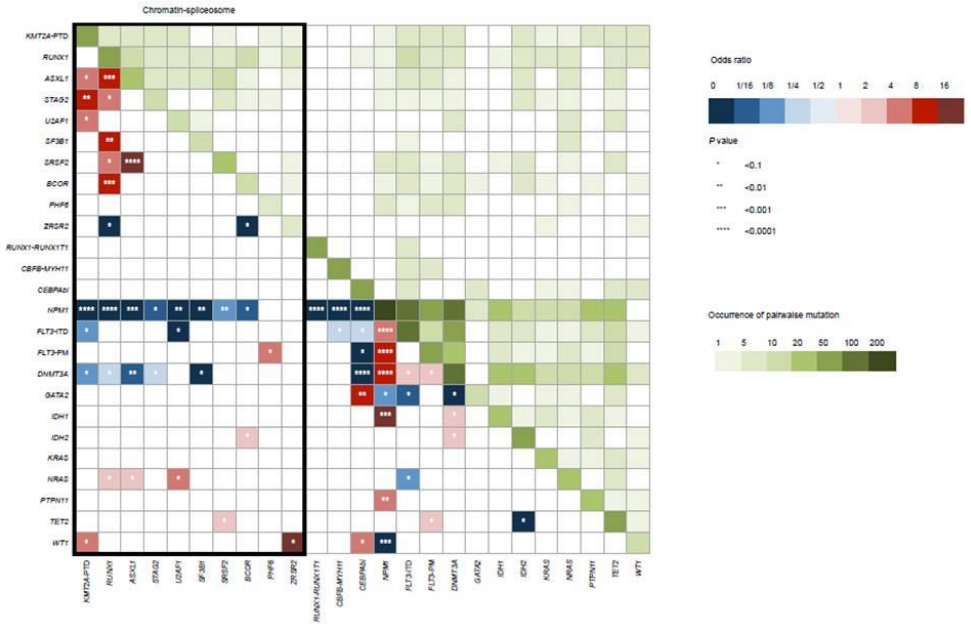


Figure 4. Patterns of co-occurrence and mutual exclusivity of gene mutations in the whole patients' cohort. In the lower triangle are shown pairwise associations between gene mutations. For each pair, odds ratios indicate an increased (>1) or decreased (<1) probability of co-occurrence between the two mutations as assessed by the Fisher exact test for statistical significance. The odds ratio of the association is color coded and the significance level is indicated by the number of asterisks in each colored square as reported in the legend at the right of the figure. Chromatin-spliceosome mutations are highlighted in the black frame. The upper triangle illustrates the absolute number of occurrences of each molecular pair, shown in green gradient and divided in intervals as reported in the legend. Mutations occurring in less than 6 patients and not defining AML categories were excluded from this analysis.

3.4.2. Outcomes after intensive induction

Patients with *de novo* AML, sAML and CS-AML were equally distributed as to the randomly assigned induction regimen (conventional ICE or high-dose sHD) and showed a comparable performance status (Table 1).

Clinical characteristics	**CS-AML N=55**	***P****	**sAML N=100**	***P****	***de novo* AML N=258**	***P*******
Age [years], median (range)	58 (20-72)	0.5542	59 (22-72)	<0.0001	48 (16-73)	0.0001
<60, n(%)	33 (60)	0.4015	53 (53)	<0.0001	207 (80.2)	0.0013
≥60, n(%)	22 (40)		47 (47)		51 (19.8)	
Gender, n (%)		0.0349		0.0076		0.8048
M	25 (45.5)		63 (63)		122 (47.3)	
F	30 (54.5)		37 (37)		136 (52.7)	
ECOG PS, n (%)		0.5674		0.4207		0.9903
0-1	49 (89.1)		92 (92)		230 (89.1)	
2-3	6 (10.9)		8 (8)		28 (10.9)	
Hepatomegaly, n (%)	5 (9.1)	0.5216	6 (6)	0.4916	21 (8.1)	0.7897
Splenomegaly, n (%)	2 (3.6)	0.2150	10 (10)	0.3159	36 (14)	0.0334
Extramedullary involvement, n (%)	6 (10.9)	0.5458	7 (7)	0.0267	41 (15.9)	0.3477
Hemoglobin [g/dL], median (range)	9.3 (5.1-13.8)	0.6334	8.8 (4.3-13.7)	0.1905	9.3 (3-15.8)	0.7040
WBC count [x10^9/L], median (range)	8.1 (1.1-252)	0.1794	4.8 (0.8-237)	<0.0001	22.3 (0.5-282)	0.0004
Platelets, median (range)	77 (12-815)	0.1441	57 (2-338)	0.3649	52 (5-852)	0.0151
BM blast cells (%), median (range)	80 (8-100)	0.0227	64 (2-100)	<0.0001	80 (0-100)	0.2067
AML with multilineage dysplasia, n (%)	6 (10.9)	0.7786	9 (9)	0.0041	5 (1.9)	0.0051
Induction treatment, n (%)		0.7613		0.6852		0.5065
ICE	25 (45.5)		48 (48)		130 (50.4)	
sHD	30 (54.5)		52 (52)		128 (49.6)	

Abbreviations: CS-AML, chromatin-splicing acute myeloid leukemia; sAML, secondary acute myeloid leukemia; *de novo* AML, *de novo* acute myeloid leukemia; ECOG PS, Eastern Cooperative Oncology Group Performance Status; WBC, white blood cell count; BM, bone marrow. *P*-values

refer to: *CS-AML vs sAML; **sAML vs *de novo* AML; *** *de novo* AML vs CS-AML. Hepatomegaly was defined as lower liver edge >2 cm from costal margin. Splenomegaly was defined as spleen >1 cm from costal margin, confirmed by ultrasound scan with longitudinal axis >12 cm. Extramedullary AML was defined as AML presenting with central nervous system involvement or mass lesions.

Table 1 Demographic and clinical characteristics by AML category.

After the first induction cycle, a lower proportion of sAML patients achieved CR (51/100, 51%) as compared to both *de novo* AML (228/258, 88.4%; *P*<0.0001) and CS-AML (43/55, 78.2%; *P*=0.002). However, also CS-AML showed a trend toward an inferior CR rate as compared to *de novo* AML (*P*=0.07). Interestingly, no significantly worse CR rate was observed between these latter groups (*P*=0.14) when accounting for both first induction (performed in all patients) and intensified re-induction (performed in patients who did not respond after first cycle). By contrast, sAML patients did not improve their CR rate (59%) even after undergoing re-induction, as compared with both *de novo* AML (92.6%, *P*<0.0001) and CS-AML (85.5%, *P*=0.001). In addition, early death more frequently occurred in sAML patients (14/100, 14%) than in *de novo* AML (11/258, 4.3%; *P*=0.003) and CS-AML (3/55, 5.5%; *P*=0.17). Globally, 41% of sAML patients, 14.5% of CS-AML patients and 7.3% of *de novo* AML patients did not achieve CR because of early death or chemo-resistance; these patients carried a dismal 1-year OS (17%) (Supplementary Figure 1).

We investigated factors affecting the probability of achieving CR (accounting for both induction cycles) by performing univariate analysis within each AML category. In *de novo* AML, a significantly negative impact was observed for advanced age, impaired performance status, high WBC and the presence of *FLT3*-ITD mutations. Apart from the presence of *SRSF2* mutations among CS-AML patients [HR 0.16 (95% CI 0.02-1.02), *P*=0.05], no other relevant clinical or biological factors (including the intensity of induction regimen) were identified for sAML and CS-AML patients (Supplementary Table 1).

3.4.3. Survival outcomes

The median follow-up for survival analysis was 4.9 years (range 0.2-8.4 years). *De novo* AML patients showed a markedly better 5-years OS (61%) and DFS (54%) than CS-AML (OS 30%, DFS 26%; *P*<0.0001 and *P*=0.0009 respectively) and sAML (OS 17%, DFS 22%; *P*<0.0001 for both comparisons) (Figure 5A and 5B). Patients with sAML carried a significantly worse OS as compared to CS-AML (*P*=0.02) (Figure 5A); however, possibly due to the high mortality rate of non-responding patients, no difference was observed between the two categories when considering only patients achieving CR,

who showed an overlapping 5-years DFS (26% and 22% respectively, *P*=0.32) (Figure 5B).

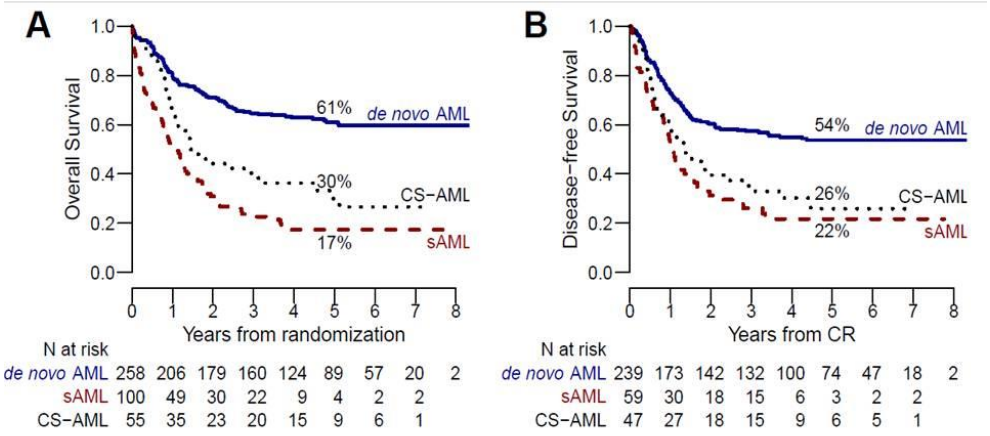


Figure 5. Kaplan-Meier survival analysis according to AML category. Survival estimates were calculated at 5 years and not censored at allogeneic transplantation. (A) Overall survival; CS-AML vs *de novo* AML, *P*<0.0001; sAML vs *de novo* AML, *P*<0.0001; CS-AML vs sAML, *P*=0.02. (B) Disease free survival; CS-AML vs *de novo* AML, *P*=0.0009; sAML vs *de novo* AML, *P*<0.0001; CS-AML vs sAML, *P*=0.32.

According to trial risk stratification criteria, a consolidative alloHSCT in first CR was administered in 18/47 (38.3%) CS-AML, 31/59 (52.5%) sAML and 80/239 (33.5%) *de novo* AML patients, at a median age of 49.5, 52 and 44.5

years, respectively. By time-dependent analysis, the administration of alloHSCT in first CR carried a 5-years survival advantage in each AML category (CS-AML: 48% vs 24%, *P*=0.07; sAML: 38% vs 8%, *P*=0.0001; *de novo* AML: 75% vs 59%, *P*=0.01) (Figure 6A-C).

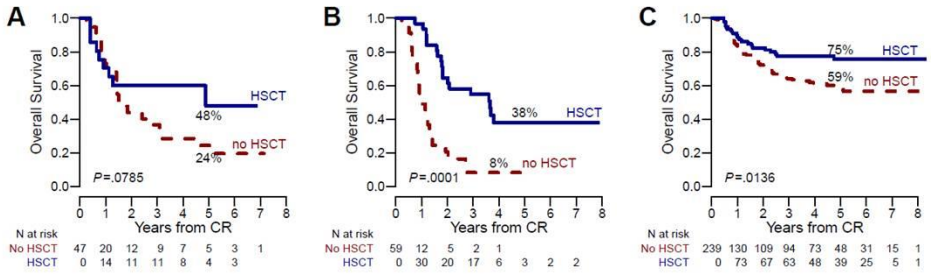


Figure 6. Simon-Makuch plots of overall survival according to allogeneic hematopoietic stem cell transplant. Transplant was considered as a time-dependent event. Survival estimates were calculated at 5 years from the date of complete remission after induction chemotherapy. (A) CS-AML, (B) sAML and (C) *de novo* AML.

By multivariable analysis performed on the whole patients' cohort accounting for age, performance status, WBC count at diagnosis and induction arm (standard vs high-dose regimen), the markedly unfavorable prognosis of the sAML category was evident for each considered outcome [CR: HR 0.09 (95% CI 0.05-0.19), *P*<0.0001; OS: HR 3.71 (95% CI 2.69-5.12), *P*<0.0001; and DFS: HR 2.54 (95% CI 1.76-3.67), *P*<0.0001)]. However, also

the CS-AML category was independently associated to a negative prognosis, in terms of OS [HR 2.2 (95% CI 1.48-3.25), *P*=0.0001] and DFS [HR 1.89 (95% CI 1.27-2.81), *P*=0.0018], but not CR (Table 2). Other factors affecting clinical outcomes included age ≥60 years, performance status (on CR and OS) and WBC ≥50x10^9/L (on OS and DFS). The administration of standard vs high-dose induction regimen did not significantly impact on clinical outcomes in the whole patients' cohort.

Since chromatin-spliceosome mutations frequently co-occurred within individual patients, we sought to investigate whether specific variants of the signature might be independently responsible for the adverse prognosis of CS-AML patients. In a multivariable analysis performed on CS-AML patients including CS-mutations and adjusting for the same variables as in previous analysis (Supplementary Table 2), only *RUNX1* and *U2AF1* independently affected OS [HR 3.55 (95% CI 1.28-9.87), *P*=0.01 and HR 6.87 (95% CI 1.71-27.55), *P*=0.006] and DFS [HR 3.13 (95% CI 1.1-8.95), *P*=0.03 and HR 16.46 (95% CI 3.14-86.31), *P*=0.0009]. Reduced DFS was observed in patients treated with the less intensive ICE induction regimen [HR 0.23 (95% CI 0.05-0.98), *P*=0.05].

All patients	Complete remission		Overall survival		Disease free survival	
Characteristics	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Age [years]						
≥60	0.38 (0.21-0.7)	0.0017	1.67 (1.25-2.23)	0.0005	1.32 (0.95-1.83)	0.1030
ECOG PS						
2-3	0.25 (0.11-0.57)	0.0010	2.32 (1.55-3.47)	<0.0001	1.46 (0.87-2.43)	0.1502
WBC count [x109/L]						
≥50	0.5 (0.24-1.03)	0.0582	1.69 (1.23-2.3)	0.0010	1.59 (1.14-2.21)	0.0063
Induction arm						
ICE	0.87 (0.48-1.57)	0.6486	1.27 (0.97-1.66)	0.0856	1.27 (0.95-1.7)	0.1046
AML category						
sAML	0.09 (0.05-0.19)	<0.0001	3.71 (2.69-5.12)	<0.0001	2.54 (1.76-3.67)	<0.0001
CS-AML	0.51 (0.2-1.37)	0.1608	2.2 (1.48-3.25)	0.0001	1.89 (1.27-2.81)	0.0018
Abbreviations: ICE, idarubicin, cytarabine and etoposide; HR, hazard ratio; CI, confidence interval.						

Table 2 Multivariable analysis for complete remission, overall survival and disease free survival on the whole patient cohort.

3.5. Discussion

In this study, we tested the possibility of implementing current clinical and cytogenetic criteria with molecular information to recognize sAML cases among otherwise defined *de novo* AML patients by a chromatin-spliceosome mutational signature (14). Importantly, the identification of patients carrying these mutations has been allowed by conventional methods and by commercially available NGS solutions, which can be easily and cost-effectively implemented in the routine diagnostic work-up of AML (18). Since diagnostic uncertainty is higher in cases lacking informative cytogenetics, we focused the search of chromatin-spliceosome mutations on patients with normal karyotype; anyway, in previous studies these mutations were infrequently associated with abnormalities of chromosomes 5 and 7 or complex karyotypes (9,19). To the best of our knowledge, this is the first report formally comparing outcomes of patients with *de novo* AML carrying this mutational profile and sAML (defined by clinical and/or cytogenetic criteria) on a large prospective cohort. Patients included into the study showed a broad age range representative of real life population, were homogeneously treated with intensive chemotherapy within a prospective clinical trial and showed a long duration of follow-up, which allowed to effectively study the prognostic relevance of chromatin-

spliceosome mutations. As an additional feature, we evaluated the impact of alloHSCT in each AML category.

In keeping with previous observations, in our study the identification of a chromatin-spliceosome mutational signature revealed markedly high-risk features in about 18% of otherwise defined *de novo* AML patients (9,14,15), which represents a not negligible proportion of the whole analyzed cohort, quite consistent with that reported by other studies (14,20). Beyond mutations in *KMT2A*-PTD, *RUNX1* and *ASXL1*, we showed that also mutations in *U2AF1* carry independent prognostic impact, indicating that the full signature might be further evaluated for assignment to the adverse prognostic group of the ELN stratification model (7). In addition, we clearly highlighted that clinical characteristics and survival outcomes of CS-AML more closely resembled those of sAML than *de novo* AML, suggesting the validity of using a molecular definition for a more comprehensive and accurate diagnosis of sAML. Since CS-AML represents a genomically and clinically homogeneous group, we think that our study provides an additional piece of evidence in favor of its recognition by the WHO classification of myeloid neoplasms (1).

The integration of clinical history, cytogenetics and molecular profile to extend the possibility of defining sAML might carry relevant implications for the appropriate clinical management of this high-risk group. As we observed a survival advantage in both sAML and CS-AML patients to whom alloHSCT was offered in first CR, patients with a chromatin-spliceosome molecular profile might be considered for intensive treatment strategies comprising rapid allocation to alloHSCT. The main challenge in this setting, however, would be the improvement of remission rates and depth to extend the access to a potentially curative alloHSCT, by exploiting innovative therapeutics possibly overcoming the inherent chemo-resistance of CS-AML. In this regard, understanding the close similitude between sAML and CS-AML should facilitate the optimization of available treatment strategies as well as the design of dedicated clinical trials. Among potentially useful agents, CPX-351 has been recently approved by FDA and EMA specifically for the treatment of AML with MDS-related changes or therapy-related AML and might provide a similar benefit in fit CS-AML patients (4). Furthermore, in a large phase 1b trial the anti-Bcl-2 agent venetoclax in association with hypomethylating agents has provided promising CR and survival rates even in sAML patients or AML with poor cytogenetics (5), while spliceosome

modulators (21,22) and DOT1L inhibitors (23) may represent a rational candidate for functional targeting of CS-AML and are currently being evaluated in clinical trials involving myeloid neoplasms. Finally, in our dataset *IDH2* mutations were reported in 20% of CS-AML patients, confirming previous observations (14) and representing another potentially important therapeutic target in this population (24).

The bottom line is that an accurate cytogenetic and molecular characterization is required at the diagnosis of AML, making reasonable to wait for these data in order to perform the best treatment decision or enrollment into clinical trials; this approach has recently demonstrated to be safe in clinically stable patients (25). In such context, although conventional cytogenetics is still needed for a correct risk stratification (7), NGS technologies may overcome its limitations and long turnaround time (18), also providing additional information with improved cost-effectiveness.

In conclusion, we have assessed the impact of a chromatin-spliceosome mutational signature on a large prospective cohort of AML patients employing a standardized, easily implementable NGS method and highlighting the need to detect this signature at diagnosis for an accurate

risk prediction. The presented data might contribute to a refined definition of the high-risk sAML category, with relevant implications for the clinical management of AML patients.

3.6. Supplementary Materials

	CS-AML (N=55)		**sAML (N=100)**		***de novo* AML (N=258)**	
Characteristics	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Age [years]						
≥60	2.22 (0.46-16.27)	0.36	0.15 (0.05-0.38)	0.06	0.45 (0.2-1.01)	0.0001
ECOG PS						
2-3	0.28 (0.04-2.32)	0.19	0.39 (0.08-1.67)	0.21	0.13 (0.05-0.36)	0.0001
WBC count [x109/L]						
≥50	0.71 (0.14-5.39)	0.70	0.54 (0.15-1.92)	0.34	0.31 (0.11-0.79)	0.01
Induction arm						
ICE	0.81 (0.17-3.79)	0.78	0.68 (0.3-1.51)	0.35	1.14 (0.44-2.96)	0.78
Genetics*						
normal karyotype	0.98 (0.05-7.05)	0.98	1.66 (0.5-6.53)	0.42	0.66 (0.21-1.8)	0.44
t(8;21)	-		-		1.93 (0.37-35.59)	0.53
inv(16)/t(16;16)	-		-		3.12 (0.61-57)	0.28
complex karyotype	-		0.55 (0.23-1.25)	0.16	-	
chromosome 7 abnormalities	-		1.66 (0.5-6.53)	0.42	-	
chromosome 5 abnormalities	-		1.04 (0.17-8.21)	0.96	-	
RUNX1-RUNX1T1	-		-		2.14 (0.41-39.36)	0.47
CBFB-MYH11	-		-		3.4 (0.67-62.06)	0.24
biallelic *CEBPα*	-		-		2 (0.38-37.03)	0.51
NPM1	-		-		0.53 (0.17-1.43)	0.23
FLT3-ITD	>99.99 (0-NA)	0.99	1.42 (0.26-10.59)	0.69	0.39 (0.15-1.02)	0.05
KMT2A-PTD	2.87 (0.59-21.02)	0.22	2.37 (0.29-49.13)	0.46	-	
ASXL1	0.67 (0.12-5.25)	0.66	0.06 (0-0.89)	0.07	-	
BCOR	>99.99 (0-NA)	1.00	-		-	
RUNX1	0.55 (0.1-2.8)	0.47	1.33 (0.1-17.65)	0.82	-	
SF3B1	>99.99 (0-NA)	0.99	-		-	

STAG2	1.07 (0.14-22.47)	0.95	-		-	
U2AF1	1.12 (0.15-23.23)	0.92	-		-	
SRSF2	0.16 (0.02-1.02)	0.05	0.06 (0-0.89)	0.07	-	

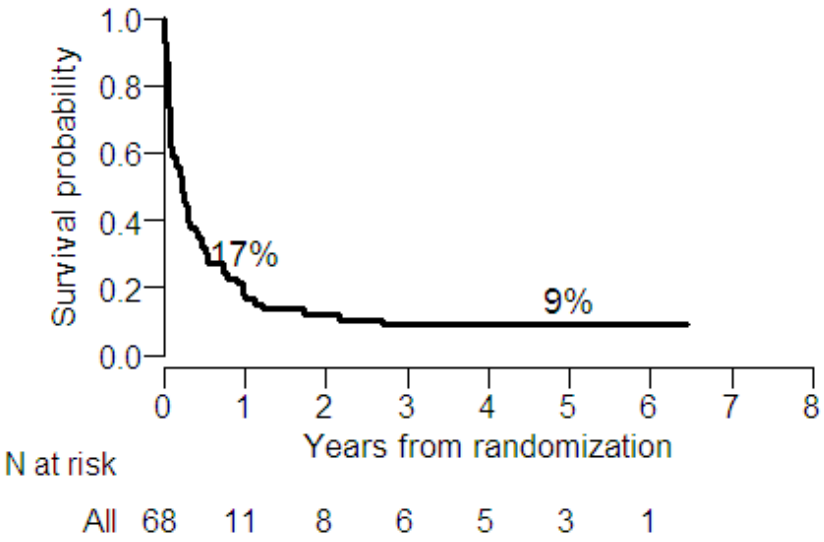
Abbreviations: ICE, idarubicin, cytarabine and etoposide; HR, hazard ratio; CI, confidence interval; NA, not applicable.

*For this analysis, we considered only chromatin-spliceosome mutations and abnormalities included in the ELN risk stratification occurring in at least 3 patients in each AML category.

Supplementary Table 1. Univariate analysis for CR by AML category on the whole patients' cohort.

CS-AML patients	Complete remission		Overall survival		Disease free survival	
Characteristics	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Age [years]						
≥60	5.73 (0.09-2224.02)	0.46	1.17 (0.34-4.00)	0.80	3.88 (0.92-16.38)	0.07
ECOG PS						
2-3	0.03 (0-32.95)	0.31	42.76 (3.22-566.79)	0.004	23.26 (0.87-622.92)	0.06
WBC count [x109/L]						
≥50	3.51 (0.02-20080.4)	0.66	0.18 (0.03-1.22)	0.08	0.06 (0.01-0.58)	0.01
Induction arm						
ICE	0.39 (0.01-7.11)	0.55	0.56 (0.21-1.48)	0.24	0.23 (0.05-0.98)	0.05
Gene mutations						
KMT2A-PTD	3.13 (0.14-115.45)	0.46	0.64 (0.21-2.00)	0.45	0.99 (0.28-3.48)	0.98
FLT3-ITD	>99.99 (0-NA)	1.00	2.21 (0.36-13.4)	0.39	3.4 (0.49-23.45)	0.21
ASXL1	5.97 (0.12-3620.83)	0.45	0.61 (0.18-2.06)	0.43	1.38 (0.36-5.21)	0.64
BCOR	>99.99 (0-NA)	1.00	0.78 (0.12-5.11)	0.79	0.73 (0.09-5.75)	0.76
RUNX1	0.09 (0-1.75)	0.14	3.55 (1.28-9.87)	0.01	3.13 (1.1-8.95)	0.03
SF3B1	>99.99 (0-NA)	1.00	0.36 (0.06-1.99)	0.24	0.2 (0.02-1.62)	0.13
SRSF2	0.16 (0-10.43)	0.42	1.50 (0.34-6.56)	0.59	0.4 (0.06-2.75)	0.35
STAG2	0.3 (0-20.86)	0.56	0.79 (0.14-4.40)	0.78	0.37 (0.05-2.97)	0.35
U2AF1	0.4 (0-38.95)	0.66	6.87 (1.71-27.55)	0.006	16.46 (3.14-86.31)	0.0009
Abbreviations: ICE, idarubicin, cytarabine and etoposide; HR, hazard ratio; CI, confidence interval.						

Supplementary Table 2. Multivariable analysis for CR, OS and DFS on 55 CS-AML patients.



Supplementary Figure 1. Kaplan-Meier analysis of OS on 68 patients not achieving CR after 1 or 2 induction cycles.

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CHAPTER 4

IMPACT OF HIGH RISK MOLECULAR MUTATIONS AFTER ALLOGENIC TRANSPLANTATION IN MYELOFIBROSIS: LONG TERM RESULTS OF A PROSPECTIVE GITMO CLINICAL TRIAL

IMPACT OF HIGH RISK MOLECULAR MUTATIONS AFTER ALLOGENIC TRANSPLANTATION IN MYELOFIBROSIS: LONG TERM RESULTS OF A PROSPECTIVE GITMO CLINICAL TRIAL

S. Salmoiraghi[1], F. Patriarca[2], A. Civini[1], C. Pavoni[1], M.C. Finazzi[1], P. Guglielmelli[3], F. Narni, D[4], E. Rumi[5], Russo[6], G. Messina[7], E. Angelucci[8], G. Milone[9], A. Imovilli[10], B. Bruno[11], M. Musso[12], P. Di Bartolomeo[13], M. Pini[14], F. Bonifazi[15], P. Corradini[16, 17], O. Spinelli[1] and A. Rambaldi[1,17]

1. ASST Papa Giovanni XXIII, Ematologia e Trapianto di Midollo Osseo
2. AOU Santa Maria della Misericordia, Clinica Ematologica
3. AOU Careggi, TMO Ematologia
4. University Hospital of Modena
5. Policlinico S. Matteo, Pavia
6. AO Spedali Civili, USD - TMO Adulti
7.Bianchi-Melacrino-Morelli" Hospital, Reggio Calabria
8. IRCSS "San Martino" Hospital, Genova
9. Ferrarotto Hospital, Catania
10. Arciospedale S. Maria Nuova, Reggio Emilia
11. Citta' della Salute e della Scienza, Torino
12. La Maddalena, UO Oncoematologia e TMO, Palermo
13. Ospedale Civile, Pescara
14. AOSS Antonio e Biagio, Alessandria
15. Institute of Hematology "Seragnoli",
16. Istituto Nazionale dei Tumori, Milano
17. Dipartimento di Oncologia e di Ematoncologia, Università degli studi di Milano

4.1. Introduction

Myelofibrosis (MF) is the most aggressive *BCR-ABL1* negative myeloproliferative neoplasm (MPNs), characterized by stem cell-derived clonal proliferation, marrow fibrosis and poor survival often associated to a leukemic progression.(1) The introduction of *JAK2* inhibitors for the treatment of MF improved the outcome of patients by reducing spleen size and constitutional symptoms, typical of the disease. However, *JAK2* inhibitors do not impact significantly on the clonal proliferation and did not allow to achieve a complete remission of the disease. Thus, the only treatment capable of eradicating MF is allogeneic hematopoietic stem cells transplantation (allo-HSCT).(2) However, this procedure is still characterized by a relevant relapse and non relapse mortality rate, even because, in patients affected by MF, HSCT is complicated by a high incidence of poor graft function or graft failure caused by both splenomegaly and by a "pro-inflammatory" marrow niche characterizing MF biology. As a consequence, it is necessary to reserve transplantation only to a minority of patients. (3) Thus, it is crucial to relay on prognostic models that facilitate treatment decision making allowing accurate risk stratification of patients in term of overall and leukemia-free survival. The prognostic evaluation of MF patients is evolving during the time together with a better understanding of the mutational landscape of the disease. Although the altered activation of the *JAK-STAT* pathway remains the hallmark of molecular pathogenesis of MPN (4), the role of genomic alterations involving genes affecting genes involved in epigenetic modification of chromatin or RNA splicing, are largely described in MF. (5) The presence of these additional genomic alterations

have an impact on the biology of the disease and some of them, affecting specific genes, have an important prognostic value.(6) In 2013 Vannucchi et al, reported a set of 5 'high molecular risk' mutations (HMR), among these non-driver mutations. The presence of at least an aberration in *ASXL1*, *EZH2*, *SRSF2,* or *IDH1/2* correlated with worse outcomes.(7) The integration of the information concerning the genomic alterations with the clinical data has recently resulted in the development of prognostic scoring systems. (8,9) In this context, the application of these novel prognostic tools should be considered to address patient to HSCT at an early stage of the disease. To date, available data regarding the application of these scoring systems in predicting the outcome of MF patients who underwent alloHSCT are still limited. Thus, in this study, following the recent publication of the new MIPSS-70 and MIPSS-70+ scores, we analyzed by sequencing the pre-transplant samples from MF patients who underwent HSCT in order to detect HMR lesions. Therefore, we evaluated the possible correlation between HMR alterations and the clinical outcome after transplantation.

4.2. Patients and Methods

4.2.1. Patients Characteristics

Molecular profile was performed on a total of 44 out of 62 patients enrolled into the GITMO-MF2010 clinical trial (ClinicalTrials.gov NCT01814475) for which pre-transplant DNA sample was available.(10) This protocol was a perspective randomized phase II trial comparing busulfan-fludarabine (BF) reduced-intensity conditioning (RIC) with thiotepa-fludarabine (FT) RIC

regimen prior to allogeneic transplantation of hematopoietic cells. The primary study endpoint was progression–free survival (PFS), assessed at 1 year after transplantation. Eligible patients were from 18 to 70 years old, affected by primary or secondary MF and with at least one unfavorable prognostic factor (hemoglobin <10 g/dL or leukocytes >25 × 10^9/L or >1% circulating blasts or constitutional symptoms). Patients characteristics were summarized in Table 1. The median age of the cohort was 56 years (range, 36 to 66 years). Twenty-one patients have been randomized in the BF arm and 23 patients in the FT arm.

Characteristics	All, n =44	BF, n=21	FT, n=23	P value
Age at randomization, median (range)	56 (36-66)	56 (41-65)	57 (36-66)	0.7866
Sex, N(%)				0.4805
F	10 (22.7)	6 (28.6)	4 (17.4)	
M	34 (77.3)	15 (71.4)	19 (82.6)	
Myelofibrosis, N(%)				0.7409
Primary	24 (54.5)	12 (57.1)	12 (52.2)	
Secondary to PV/ET	20 (45.5)	9 (42.9)	11 (47.8)	
Bone marrow fibrosis grade >=2	37 (86)	16 (80)	21 (91.3)	0.3929
Peripheral blood blast count >=2%, n(%)	18 (42.9)	10 (50)	8 (36.4)	0.3725
Platelets < 100 x 109/L, N(%)	24 (54.5)	12 (57.1)	12 (52.2)	0.7409
Hemoglobin <100 g/L	33 (75)	15 (71.4)	18 (78.3)	0.6011
Leucocytosis >25x109/L	3 (6.8)	1 (4.8)	2 (8.7)	1
Constitutional symptoms	23 (52.3)	12 (57.1)	11 (47.8)	0.5366
Comorbidity score >=3, N(%)	13 (29.5)	4 (19)	9 (39.1)	0.1447
Splenomegaly, N(%)	33 (75)	15 (71.4)	18 (78.3)	0.6011
Splenectomy, N(%)	8 (18.2)	4 (19)	4 (17.4)	1
Donor				0.7862
Sibling	18 (40.9)	8 (38.1)	10 (43.5)	
Unrelated matched	18 (40.9)	10 (47.6)	8 (34.8)	
Unrelated mismatched	8 (18.2)	3 (14.3)	5 (21.7)	
Source of HSC				0.6575

BM	5 (11.4)	3 (14.3)	2 (8.7)	
PB	39 (88.6)	18 (85.7)	21 (91.3)	
Karyotype, N(%)				0.1569
Normal	15 (34.1)	4 (19)	11 (47.8)	
Unfavorable alterations	8 (18.2)	5 (23.8)	3 (13)	
Other alterations	5 (11.4)	4 (19)	1 (4.3)	
Unknown	16 (36.4)	8 (38.1)	8 (34.8)	

Table 1. Patients characteristics according to randomization arm

4.2.2. Methods

To analyze the pre-transplant prospectively collected DNA samples, we applied a Next Generation Sequencing (NGS) panel selecting targeted gene regions related to myeloid neoplasms. This panel is commercially available NGS library preparation kit (Sophia Myeloid Solution by SOPHiA GENETICS, SA, CH) allowing to obtain DNA libraries for sequencing to investigate 30 myeloid neoplasms related genes listed in Table 2. The libraries were sequenced and demultiplexed on a MiniSeq instrument (Illumina, San Diego, CA). The aligned sequences were analyzed to identify differences with the reference genome (GRCh37/hg19) with the detection limit/sensitivity (LOD) down to 2.5% variant allele frequency (VAF), as recommended by the producer. This condition guaranteed the identification of mutation with a low VAF, therefore to molecularly characterize also minor clones. Variant calling and predictions of functional effects of the mutants were performed by the use of SOPHiA DDM™ software which takes into consideration different biologic databases available online. Frameshift and nonsense variants were always considered as having an impact on the functionality. Single nucleotide variants and in-frame indels were retained in the absence

of description as genetic polymorphism into public databases of human polymorphisms (GnomAD and ddSNP). For alterations of splicing sites and splicing related regions, we used the Human Splicing tool (http://www.umd.be/HSF3) to predict the effect on the splicing process. Finally, the description in other cancer specimens of the identified mutations was checked against COSMIC database (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic).

The achievement of full donor chimerism (FDC) (defined as >95% of cells being of donor origin) was evaluated by molecular analysis of short tandem repeats on bone marrow cells and PB mononuclear cells, collected at day +30, day +100, day +180, 1 year, and 2 years after transplant procedure.

Gene partially sequenced	Gene entirely sequenced
ABL1 (4-9)	*CEBPA*
ASXL1 (9,11,12,14)	*CSF3R*
BRAF (15)	*DNMT3A*
CALR (9)	*ETV6*
CBL (8,9)	*EZH2*
FLT3 (13-15,20)	*JAK2*
HRAS (2,3)	*RUNX1*
IDH1 (4)	*TET2*
IDH2 (4)	*ZRSR2*
KIT (2,8-11,13,17,18)	
KRAS (2,3)	
MPL (10)	
NPM1 (10,11)	
NRAS (2,3)	
PTPN11 (3,7-13)	
SETBP1 (4)	
SF3B1 (10-16)	
SRSF2 (1)	
TP53 (2-11)	
U2AF1 (2,6)	
WT1 (6-10)	

Table 2: Target gene for sequencing selected by Sophia Myeloid Solution by SOPHiA GENETICS

The clinical endpoints included in the analysis were Overall Survival (OS), Progression-free Survival (PFS), Non Relapse Mortality (NRM), Cumulative Incidence of Relapse (CIR) and engraftment. OS was calculated from transplant to death. PFS, NRM and CIR were calculated from transplant to relapse or death, whichever occurred first. Neutrophil engraftment was defined as the number of days after transplantation taken to achieve an absolute neutrophil count of at least $0{\cdot}5 \times 10^9$ cells/L and platelet

engraftment was defined as the number of days to maintain an untransfused platelet count of at least 20·0 × 10^9 cells/L.

Baseline continuous characteristics were presented as median with range; categorical variables were reported with absolute and percentage frequencies. PFS and OS were estimated using Kaplan-Meier method and log-rank test was applied to test differences between groups. NRM and CIR were estimated using cumulative incidence function, considering relapse and death as a competing event, respectively, and the Fine and Gray's non-parametric test was used to assess group differences. The univariate analyses were performed by fitting Cox models and HR with 95% confidence intervals were reported. All reported p-values were two sided and the conventional 5% significance level was fixed. All analyses were done with R software (version 4.0.0).

4.3. Results

4.3.1. Genetic variants identified in MF pre-transplant samples

Analyzing the sequences, we identified a total of 112 variants considered as pathogenic or likely pathogenic, including non-synonymous point mutations (missense (n=75) or nonsense (n=13)), small insertions or deletions (in frame (n=3) or frame-shift (n=20)) and splicing sites mutations (n=1). Of the 30 genes included in the panel, 19 of them were mutated in at least one patient. Figure 1 shows these 19 genes grouped by functional characteristics. Moreover, the picture indicates frequencies of mutations in the cohort and the co-occurrence with other mutations in each patient.

As expected, the most frequently mutated gene was *JAK2* (n=32 mutations, in 70% of the patients). In the great part of the cases (n=29) the alteration was the *JAK2* V617F, with a variable VAF (range 2.5-98). Three *JAK2* alterations differed from V617F, but in 2 cases these molecular lesions co-occurred with a V617F alteration and, in the remaining one, the non-canonical *JAK2* alteration co-occurred with a *MPL* mutation. Furthermore, 91% of patients (n=40) carried one of the 3 driver mutations (*JAK2V617F/CALR/MPL*, n=29, n=8, n=3). Among them, a half of *CALR* mutated patients had a favorable type 1 mutation. In line with what previously published, the remaining 9% are defined triple-negative MF (n=4). Interestingly, among these triple-negative patients, one of them did not harbor any molecular mutation in the investigated genes with a VAF>2,5%. Moreover, we identified 5 mutations in *TP53* gene: 2 were in the same patient, as frequently described for oncogenes, while the other 3 alterations were described as likely pathogenic and with a VAF around 50%. This VAF level could suggest a possible germline origin that we can not verify because of the unavailability of non cancer biologic material.

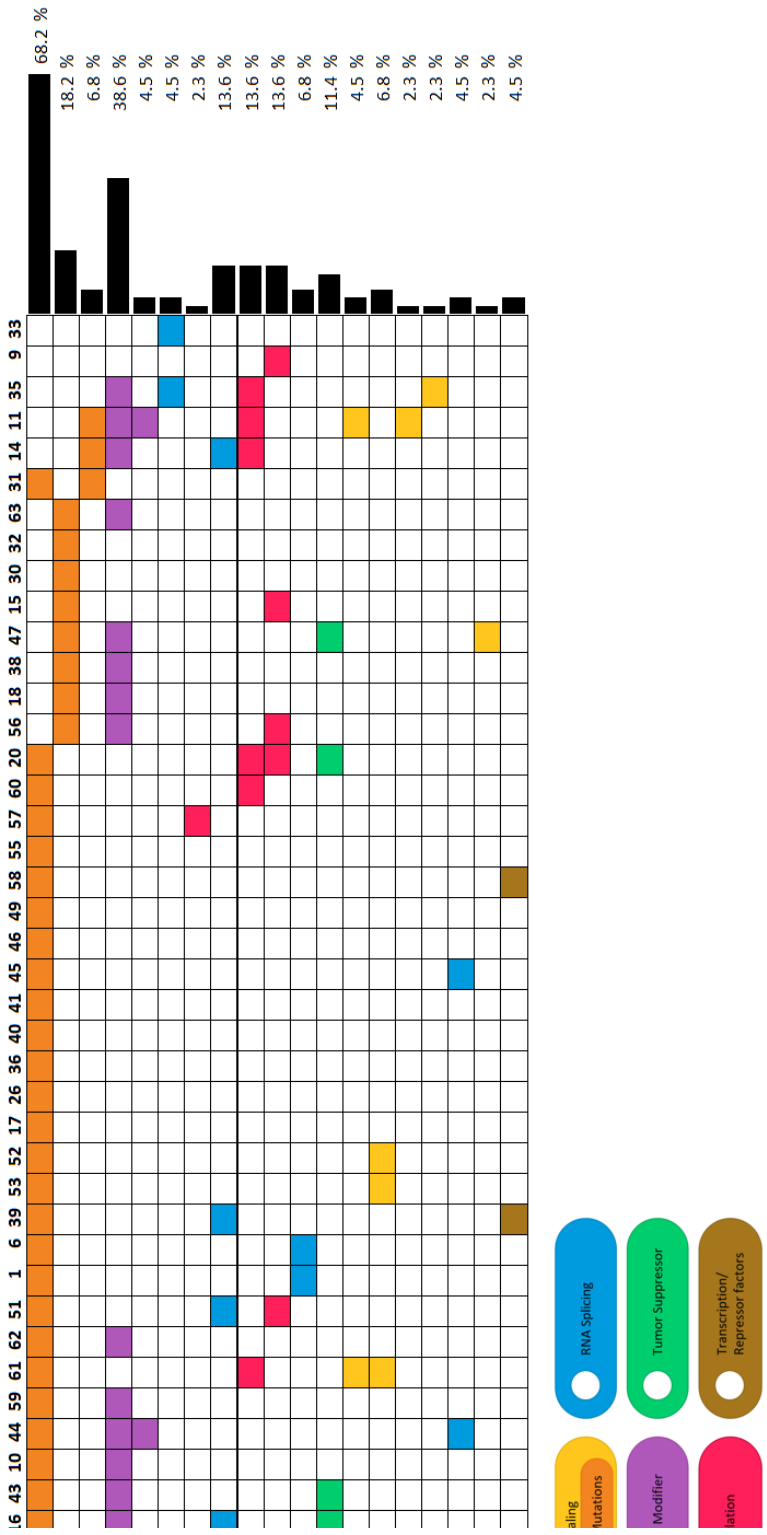

Figure 1: In this figure every column represents a patient and the number on the top of the column is their ID number. Rows indicate single genes found mutated in our cohort differently colored basing on functionality. Histograms at the end of each row indicates the cumulative incidence of gene mutations. Patient negative for all the mutation was excluded from this figure

4.3.2. HRM mutations and MIPSS scores calculation

In our investigation, we particularly focused on the identification of mutations in five genes *ASXL1, SRSF2, EZH2, IDH1, and IDH2*, considered high molecular risk mutations (HMR) within the prognostic MIPSS70 scores, as previously described. (11,12) About 43% of patients had at least 1 HMR mutated gene (n=19) while only 3 patients, accounting for 7%, were positive for two or more HRM mutations. The most frequent HMR mutated gene was *ASXL1*, while no patients carried mutations in *IDH2* gene. Furthermore, MIPSS70-plus considers *U2AF1*Q157 as an additional HMR mutation. These mutations were present in 7% (n=3) of patients.

Table 3 summarizes the classification of our cohort of patients by the different scoring systems. Thirty-eight patients (88%) had a Dynamic International Prognostic Scoring System (DIPPS) plus risk score of intermediate-2 or higher. Basing on the integration of molecular and cytogenetic data, we were able to classify our patients according to MIPSS70 scores. According to these scores, we can observe that 74% of the patients resulted high risk patients by MIPSS score, while 78% of the patients were high or very high risk by MIPSS+.

Risk categories	N patients
IPSS, N(%)	
Intermediate-I	9 (20.5)
Intermediate-II	16 (36.4)
High	19 (43.2)
DIPSS, N (%)	
Intermediate-I	12 (27.3)
Intermediate-II	30 (68.2)
High	2 (4.5)

DIPSS Plus, N (%)	
Intermediate-I	5 (11.4)
Intermediate-II	20 (45.5)
High	19 (43.2)
MIPSS (n=44)	
Low	1 (2.4)
Intermediate	10 (23.8)
High	31 (73.8)
MIPSS Plus (n=34)	
Low	3 (11.1)
Intermediate	3 (11.1)
High	15 (55.6)
Very high	6 (22.2)

Table 3: Classification of our cohort of patients by the different scoring systems.

4.3.3. Post-Transplant Outcomes

4.3.3.1. Overall Survival

The median duration of follow up for the cohort was 4.2 years (range 0.02-9). The 5-year overall survival (OS) was 60% 61% (95% CI, 48%-77%). The presence of HMR mutations or the MIPSS-70 risk class did not affected survival, since the 5-year OS for patients with or without HMR mutations was respectively 62% and 60% (p=0.77) (Figure 2) and the 5-year OS for patients in the low/intermediate and high/very high risk group was respectively 64% and 58% (p=0.45) (Figure 2). In a univariate analysis, none of the clinical or molecular factors considered influenced OS (Table 4), in particular the HRM category did not impact on survival (HR 1.15, p=0.77), as well as the presence of an unfavorable karyotype (HR 0.91, p=0.88). Furthermore, the analysis of individual mutations did not reveal any impact on survival.

4.3.3.2. Progression-free survival

The 5-year progression free survival (PFS) was 45% (95% CI, 32%-62%). PFS was not influenced by the presence of HMR mutations, with a 5-year PFS of 47% for patients without HMR and a PFS of 42% for patients harboring the HMR mutations (p=0.89) (Figure 2). Regarding the MIPPS-70 classification, there was a trend for a better PFS for patients in the low/intermediate risk patients (5-year PFS 64%) compared to patients in the high risk group (5-year PFS 38%), though not statistically significant (p=0.07) (Figure 2). The univariate analysis did not identify any clinical, cytogenetic or molecular factor influencing PFS, even if a high probability of disease progression seemed to be correlated to the presence of *NRAS* mutation (Table 4).

Factors	**Overall Survival**			**Progression Free Survival**		
	N death (%)	**HR**	**P**	**N events (%)**	**HR**	**P**
Splenomegaly	14 (42.4)	1.33 (0.47-3.79)	0.5886	20 (60.6)	1.46 (0.55-3.9)	0.4508
Splenectomy	4 (50)	0.88 (0.29-2.67)	0.8225	4 (50)	0.86 (0.29-2.5)	0.7765
Donor						
Sibling	4 (22.2)	1		8 (44.4)	1	
Unrelated matched	11 (61.1)	3.39 (1.08-10.68)	0.0366	12 (66.7)	2.12 (0.87-5.21)	0.1
Unrelated mismatched	4 (50)	2.71 (0.67-10.85)	0.16	5 (62.5)	1.84 (0.6-5.64)	0.2867
Source PB	17 (43.6)	1.04 (0.24-4.57)	0.9548	22 (56.4)	1.04 (0.31-3.49)	0.9489
Fibrosis grade >=2	15 (40.5)	0.56 (0.19-1.7)	0.3093	20 (54.1)	0.71 (0.24-2.07)	0.5253
PB blast >=2%	8 (44.4)	0.97 (0.39-2.44)	0.9546	9 (50)	0.81 (0.36-1.86)	0.6258
Platelets < 100	10 (41.7)	1.08 (0.44-2.67)	0.8667	14 (58.3)	1.05 (0.48-2.32)	0.9026
Hemoglobin <100	13 (39.4)	0.59 (0.22-1.59)	0.2941	19 (57.6)	1.03 (0.41-2.58)	0.9554
Constitutional symptoms	10 (43.5)	1.24 (0.5-3.08)	0.6418	13 (56.5)	1.24 (0.56-2.73)	0.5993

Unfavorable karyotype	4 (50)	0.91 (0.28-2.97)	0.8805	5 (62.5)	0.83 (0.29-2.33)	0.7174
HMR category	9 (47.4)	1.15 (0.46-2.82)	0.7677	11 (57.9)	1.06 (0.48-2.33)	0.8932
≥2 HMR genes	1 (33.3)	0.71 (0.09-5.33)	0.7386	1 (33.3)	0.49 (0.07-3.61)	0.4827
MIPSS (n=28)						
Low/intermediate	4 (36.4)	1		4 (36.4)	1	
High	15 (48.4)	1.47 (0.48-4.46)	0.4972	20 (64.5)	2.24 (0.77-6.56)	0.141
MIPSS Plus (n=20)						
Low/intermediate	1 (16.7)	1		2 (33.3)	1	
High	8 (53.3)	4.07 (0.5-32.91)	0.1886	12 (80)	4.05 (0.9-18.25)	0.0685
Very high	4 (66.7)	4.72 (0.52-42.46)	0.1664	4 (66.7)	3.24 (0.58-18.25)	0.1818
***JAK2*V617F**						
WT	5 (33.3)	1		6 (40)	1	
Vaf < 50	5 (50)	2.06 (0.59-7.19)	0.258	6 (60)	1.86 (0.6-5.8)	0.2836
Vaf >50	9 (47.4)	1.53 (0.51-4.63)	0.449	13 (68.4)	1.98 (0.75-5.26)	0.1691
ASXL1	9 (52.9)	1.41 (0.57-3.48)	0.4505	10 (58.8)	1.13 (0.51-2.52)	0.7661
CALR	2 (25)	0.57 (0.13-2.49)	0.4585	3 (37.5)	0.54 (0.16-1.81)	0.3196
TET2	4 (66.7)	1.72 (0.57-5.24)	0.338	4 (66.7)	1.49 (0.51-4.36)	0.4646
DNMT3A	3 (50)	0.98 (0.29-3.39)	0.978	4 (66.7)	1.09 (0.37-3.19)	0.8702
U2AF1	4 (66.7)	1.51 (0.5-4.58)	0.4643	4 (66.7)	1.38 (0.47-4.02)	0.5593
TP53	3 (60)	1.73 (0.49-6.09)	0.3904	4 (80)	1.63 (0.55-4.83)	0.3772
MPL	2 (66.7)	1.16 (0.26-5.14)	0.8456	2 (66.7)	1.29 (0.3-5.49)	0.7289
NRAS	2 (66.7)	3.39 (0.77-14.95)	0.1076	3 (100)	3.3 (0.98-11.17)	0.0545
Triple Negative	1 (25)	0.44 (0.06-3.35)	0.4288	1 (25)	0.32 (0.04-2.38)	0.2672
VAF driver*						
Vaf < 50	8 (40)	1		10 (50)	1	
Vaf >50	10 (50)	1.07 (0.42-2.72)	0.88	14 (70)	1.56 (0.69-3.54)	0.29
Treatment						
BU-FLU	10 (47.6)	1		12 (57.1)	1	
THIO-FLU	9 (39.1)	0.76 (0.3-1.88)	0.5478	13 (56.5)	0.91 (0.41-2)	0.8108

Table 4: Univariate analysis on Overall Survival (OS) and Progression Free Survival (PFS) and different clinical/biological factors. *Triple negative patients are excluded from the analysis.

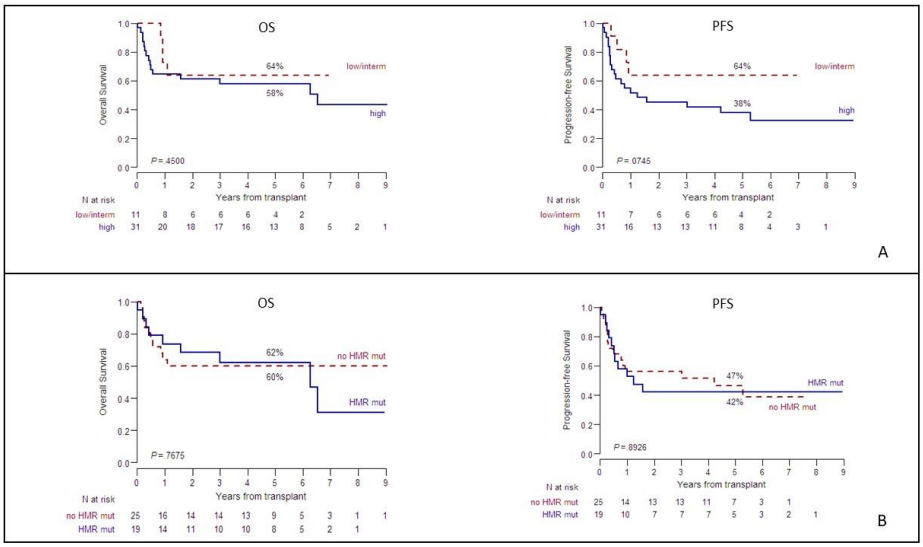

Figure 2: Panel A: OS e PFS according to MIPSS-70 risk classification, Panel B: OS e PFS according to presence of HRM mutations

4.3.3.3. Non-relapse mortality

The 5-year non relapse mortality (NRM) was 25% (95% CI, 13%-39%). NRM was not affected by the presence of HMR mutations nor by the MIPSS-70 classification, with a 5-year NRM of 26% and 24% respectively for patients with or without HRM mutations (p=0.82), and a 5-year NRM of 18% and 29% for patients respectively in the MIPSS-70 low/intermediate and high risk class (p=0.41). (Figure 3) In a univariate analysis NRM was negatively influenced by MIPPS70-plus high and very high risk class (p<0.001). There was a trend for worse NRM for patients harboring the *TP53* mutation (HR 2.86, p=0.06), whereas the presence of other mutations did not impact on NRM. (Table 5)

4.3.3.4. Cumulative Incidence of Relapse

The cumulative incidence of relapse (CIR) at 5 years was 30%. CIR was not affected by the presence of HMR mutations nor by the MIPSS-70 classification, with a 5-year CIR of 32% and 29% respectively for patients with or without HRM mutations (p=0.98), and a 5-year CIR of 18% and 33% for patients respectively in the MIPSS-70 low/intermediate and high risk class (p=0.36) (Figure 3). In a univariate analysis, no impact on CIR was observed for HMR category, unfavorable karyotype, MIPPS-70 or MIPPS-70 plus category or the presence of individual mutations. Interestingly, risk of relapse was negatively influenced by a high allele burden (>50%) characterizing driver mutations (HR 5.01, p=0.032). (Table 5) Chimerism analysis showed that a mixed chimerism is more frequent and prolonged in patients with a *JAK2* with a high allele burden.

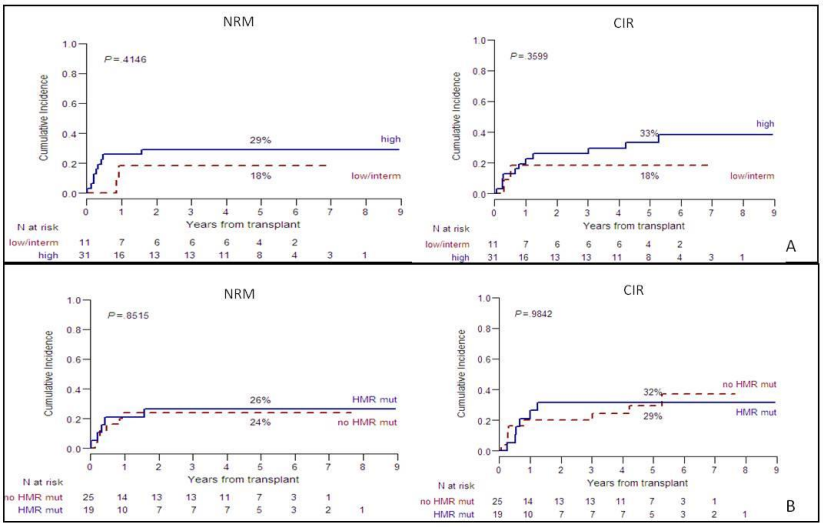

Figure 3: Panel A: NRM e CIR according to MIPSS-70 risk classification, Panel B: NRM e CIR according to presence of HRM mutations

Factors	Cumulative incidence of relapse			Non Relapse Mortality		
	N events (%)	HR	P	N events (%)	HR	P
Splenomegaly	10 (30.3)	0.76 (0.23-2.48)	0.65	10 (30.3)	3.82 (0.52-28.21)	0.19
Splenectomy	4 (50)	2.36 (0.71-7.81)	0.16	0 (0)	0 (0-0)	0
Donor						
Sibling	4 (22.2)	1		4 (22.2)	1	
Unrelated matched	8 (44.4)	2.39 (0.76-7.5)	0.13	4 (22.2)	1.13 (0.29-4.47)	0.86
Unrelated mismatched	2 (25)	1.24 (0.24-6.45)	0.8	3 (37.5)	1.89 (0.47-7.57)	0.37
Source PB	13 (33.3)	1.75 (0.23-13.26)	0.59	9 (23.1)	0.62 (0.17-2.31)	0.48
Fibrosis grade >=2	11 (29.7)	0.93 (0.21-4.06)	0.93	9 (24.3)	0.64 (0.13-3.05)	0.57
PB blast >=2%	3 (16.7)	0.36 (0.1-1.3)	0.12	6 (33.3)	1.75 (0.55-5.57)	0.34
Platelets < 100	7 (29.2)	0.73 (0.26-2.03)	0.55	7 (29.2)	1.6 (0.48-5.26)	0.44
Hemoglobin <100	12 (36.4)	2.05 (0.45-9.31)	0.35	7 (21.2)	0.58 (0.18-1.85)	0.36
Constitutional symptoms	7 (30.4)	1.01 (0.37-2.79)	0.98	6 (26.1)	1.18 (0.37-3.74)	0.78
Unfavorable karyotype	3 (37.5)	0.89 (0.26-3.02)	0.86	2 (25)	0.92 (0.2-4.23)	0.92
HMR category	6 (31.6)	0.99 (0.35-2.79)	0.99	5 (26.3)	1.12 (0.35-3.57)	0.85
≥2 HMR genes	0 (0)	0 (0-0)	0	1 (33.3)	1.44 (0.2-10.55)	0.72
MIPSS (n=28)						
Low/intermediate	2 (18.2)	1		2 (18.2)	1	

High	11 (35.5)	2.01 (0.44-9.11)	0.37	9 (29)	1.85 (0.44-7.69)	0.4
MIPSS Plus (n=20)						
Low/intermediate	2 (33.3)	1		0 (0)	1	
High	7 (46.7)	1.65 (0.38-7.17)	0.51	5 (33.3)	>99.99 (>99.99->99.99)	0
Very high	2 (33.3)	1.16 (0.19-7)	0.87	2 (33.3)	>99.99 (>99.99->99.99)	0
***JAK2*V617F**						
WT	2 (13.3)	1		4 (26.7)	1	
Vaf < 50	2 (20)	1.63 (0.23-11.6)	0.63	4 (40)	1.63 (0.41-6.49)	0.49
Vaf >50	10 (52.6)	5.26 (1.15-24.1)	0.032	3 (15.8)	0.52 (0.12-2.23)	0.38
ASXL1	5 (29.4)	0.88 (0.3-2.58)	0.81	5 (29.4)	1.39 (0.44-4.43)	0.58
CALR	1 (12.5)	0.29 (0.04-1.98)	0.21	2 (25)	1.12 (0.22-5.6)	0.89
TET2	2 (33.3)	1.22 (0.22-6.63)	0.82	2 (33.3)	1.37 (0.34-5.59)	0.66
DNMT3A	3 (50)	1.82 (0.54-6.16)	0.33	1 (16.7)	0.56 (0.08-3.72)	0.55
U2AF1	2 (33.3)	1.21 (0.24-6.13)	0.82	2 (33.3)	1.31 (0.33-5.16)	0.7
TP53	1 (20)	0.6 (0.08-4.48)	0.62	3 (60)	2.86 (0.97-8.46)	0.058
MPL	1 (33.3)	1.17 (0.12-11.22)	0.89	1 (33.3)	1.29 (0.21-7.82)	0.78
NRAS	2 (66.7)	2.69 (0.67-10.89)	0.17	1 (33.3)	1.69 (0.17-16.38)	0.65
Triple Negative	0 (0)	0 (0-0)	0	1 (25)	1 (0.13-7.48)	1
VAF driver						
Vaf < 50	3 (15)	1		7 (35)	1	
Vaf >50	11 (55)	5.03 (1.46-17.36)	0.01	3 (15)	0.36 (0.1-1.32)	0.12
Treatment						
BU-FLU	7 (33.3)	1		5 (23.8)	1	
THIO-FLU	7 (30.4)	0.81 (0.29-2.25)	0.69	6 (26.1)	1.16 (0.36-3.69)	0.8

Table 5: Univariate analysis on Cumulative Incidence of Relapse (CIR) and Non Relapse Mortality (NRM) and different clinical/biological factors

4.3.3.5. Engraftment

Neutrophils engraftment was obtained by all but 4 patients. Two patients had neutrophils value never below 0.5 x10^9/L. The median neutrophils time to engraftment was 17 days after transplant (range 10-30 days). In a univariate analysis, the probability to reach neutrophils engraftment was

reduced by MIPSS-70 high risk category (HR 0.47, p=0.041). The HMR genes category did not influence neutrophils engraftment (HR 1.72, p=0.063). Regarding the single mutations impact on neutrophils engraftment, the presence of *JAK2V617F*, *CALR*, *DNMT3A* and *NRAS* mutations reduced the probability to engraft (Table 6).

Platelets engraftment (defined as platelet number > 20.000/mcl) was not reached by 5 patients. Four patients had platelet count never below 20.000/mcl. The median platelet time to engraftment was 19 days (range 12-69 days). In a univariate analysis, splenomegaly, the high risk MIPPS-70 class, the presence of driver mutation with a VAF<50% and the treatment allocation to FT arm were associated with a reduced platelet engraftment (Table 6).

The multivariate analysis showed that splenectomy positively impacted on the engraftment of both neutrophils and platelets. Moreover, the multivariate analysis also demonstrated a significant correlation between a better neutrophils engraftment and the presence of a mutation in *CALR*, whereas *NRAS* lesions worsened the probability of neutrophils to engraft. On the other hand, the presence of *JAK2*-V617F alteration at a low burden (<50%) was negatively associated to platelets engraftment, even by multivariate analysis (Table 7).

Factors	**Neutrophil engraftment**			**Platelet engraftment**		
	N events (%)	**HR**		**N events (%)**	**HR**	
Splenomegaly	28 (87.5)	0.46 (0.25-0.83)	0.0097	27 (84.4)	0.32 (0.14-0.72)	0.006
Splenectomy	7 (100)	3.07 (1.56-6.03)	0.0011	5 (100)	3.05 (1.03-9.04)	0.044
Donor						
Sibling	17 (100)	1		16 (94.1)	1	

Unrelated matched	15 (83.3)	0.82 (0.46-1.47)	0.51	13 (81.2)	0.66 (0.3-1.41)	0.28
Unrelated mismatched	6 (85.7)	0.76 (0.28-2.03)	0.58	6 (85.7)	0.44 (0.18-1.08)	0.073
Source PB	34 (89.5)	1.35 (0.63-2.86)	0.44	31 (86.1)	0.85 (0.44-1.65)	0.63
Fibrosis grade >=2	32 (91.4)	1.06 (0.39-2.93)	0.9	30 (88.2)	1.05 (0.41-2.71)	0.91
PB blast >=2%	15 (88.2)	1.05 (0.57-1.91)	0.88	14 (87.5)	0.86 (0.45-1.66)	0.66
Platelets < 100	20 (87)	0.53 (0.3-0.93)	0.028	19 (82.6)	0.26 (0.13-0.54)	0.0003
Hemoglobin <100	29 (87.9)	0.49 (0.24-1.04)	0.063	27 (84.4)	0.51 (0.25-1.04)	0.065
Constitutional symptoms	19 (86.4)	0.78 (0.45-1.36)	0.38	17 (81)	0.5 (0.27-0.94)	0.03
Unfavorable karyotype	8 (100)	1.22 (0.63-2.37)	0.55	8 (100)	1.92 (0.9-4.11)	0.092
HMR category	17 (94.4)	1.79 (0.99-3.21)	0.053	15 (88.2)	1.17 (0.62-2.21)	0.63
≥2 HMR genes	3 (100)	3.24 (1.35-7.78)	0.0085	3 (100)	1.68 (0.72-3.92)	0.23
MIPSS (n=28)						
Low/intermediate	10 (100)	1		9 (100)	1	
High	26 (86.7)	0.47 (0.22-1.01)	0.052	24 (82.8)	0.34 (0.15-0.81)	0.015
MIPSS Plus (n=20)						
Low/intermediate	6 (100)	1		5 (100)	1	
High	12 (80)	0.44 (0.16-1.2)	0.11	11 (73.3)	0.55 (0.21-1.46)	0.23
Very high	6 (100)	0.75 (0.27-2.09)	0.58	6 (100)	1.59 (0.59-4.32)	0.36
JAK2						
WT	14 (93.3)	1		13 (92.8)	1	
VAF < 50	7 (70)	0.24 (0.1-0.58)	0.0016	7 (70)	0.27 (0.13-0.56)	0.0004
VAF >50	17 (100)	0.68 (0.35-1.31)	0.24	15 (93.8)	1.02 (0.48-2.15)	0.96
ASXL1	15 (93.8)	1.71 (0.94-3.12)	0.077	14 (93.3)	1.6 (0.89-2.87)	0.12
CALR	7 (87.5)	3.47 (1.74-6.92)	0.0004	7 (87.5)	1.59 (0.79-3.19)	0.19
TET2	5 (83.3)	1.07 (0.35-3.31)	0.9	4 (80)	0.52 (0.23-1.21)	0.13
DNMT3A	6 (100)	2.31 (1.33-4.03)	0.0031	6 (100)	1.98 (0.83-4.71)	0.12
U2AF1	5 (100)	0.98 (0.53-1.8)	0.94	4 (100)	0.75 (0.43-1.3)	0.31
TP53	4 (100)	0.71 (0.38-1.33)	0.28	4 (100)	0.77 (0.42-1.42)	0.4
MPL	3 (100)	0.99 (0.45-2.16)	0.98	2 (100)	1.07 (0.63-1.81)	0.81
NRAS	1 (33.3)	0.12 (0.02-0.76)	0.024	1 (33.3)	0.2 (0.03-1.6)	0.13
Triple Negative	4 (100)	1.52 (0.67-3.49)	0.32	4 (100)	1.7 (0.85-3.39)	0.14
VAF driver						
VAF < 50	16 (80)	1		16 (80)	1	
VAF>50	18 (100)	1.52 (0.83-2.79)	0.17	15 (93.8)	2.1 (1.01-4.39)	0.048
Treatment						
BU-FLU	19 (100)	1		17 (100)	1	

THIO-FLU	19 (82.6)	1.08 (0.62-1.9)	0.79	18 (78.3)	0.63 (0.34-1.15)	0.13

Table 5: Univariate analysis on neutrophils and platelets engraftment and different clinical/biological factors

Factors	**Neutrophil engraftment**		**Platelet engraftment**	
	HR	**P**	**HR**	**P**
Splenomegaly	0.90 (0.39-2.07)	0.80	1.09 (0.53-2.22)	0.81
Splenectomy	2.53 (1.05-6.13)	0.04	3.77 (1.59-8.97)	0.002
MIPSS (n=28)				
Low/intermediate	1		1	
high	0.87 (0.44-1.70)	0.68	0.64 (0.25-1.59)	0.34
JAK2				
WT	1		1	
VAF <50	0.52 (0.17-1.55)	0.24	0.33 (0.11-0.95)	0.04
VAF >50	1.38 (0.60-3.15)	0.45	0.93 (0.45-1.93)	0.85
CALR	3.84 (1.36-10.88)	0.01		
DNMT3A	1.49 (0.79-2.79)	0.22		
NRAS	0.16 (0.04-0.64)	0.009		

Table 6: Multivariate analysis on neutrophils and platelets engraftment and clinical and biological factors resulted significantly associated in univariate analysis

4.4. Discussion and Conclusions

Cytogenetics and molecular genetics have been recently integrated with clinical aspects to develop scoring systems for risk stratification for transplantation-age patients. Although molecular profiling has identified ruxolitinib-treated patients with decreased time to failure of *JAK2* inhibitor (13), data concerning the impact of molecular alterations as prognostic tool for the post-transplant outcome is still limited. Therefore, we performed a retrospective evaluation of post transplant outcome according to the MIPSS70 scores of patients enrolled in a GITMO perspective clinical trial, comparing 2 Reduce Intensity Conditioning (RIC) regimens.

Target gene sequencing allowed us to classify patients included in the trial by MIPSS scoring systems and we observed that the great part of them (more than 70%) resulted high or very high risk patients. Of note, only a very small number of patients selected for transplant procedure were re-classified as low risk by MIPSS score. Thus, exclusively clinical-based scoring systems (DIPSS score) used for the evaluation of the patients at the enrollment and MIPSS scoring systems are sometimes discordant, as previously described.(8)

The presence of mutations associated with worse outcomes in patients affected by MF (HRM mutations), as well as MIPSS risk classification did not impact on OS or PFS in this cohort of patients who underwent allo-HCT. Similarly, the number of mutations per patient did not affect both OS and PFS. These findings suggest that transplantation can overcome the poor prognosis associated with these mutations. These observations are in line with recent findings by Tamari (14) and can result in an accurate application of molecular based scoring systems to identify patients who should be referred to a earlier evaluation for transplant procedure. In our cohort of patients, analysis of the impact of individual mutation did not result relevant in predicting OS, even if *NRAS* alteration seemed to be associated to a high probability of MF progression. This is in contrast with other previous reports present in literature in which *ASXL1*, *IDH2* and *DNMT3A* were associated with progression and impaired survival whereas *U2A*F1 was associated to a higher risk of death due to a graft failure.(15) (14) Although we cannot draw any conclusions about the relationship between alteration in *U2AF1* and post-transplant outcome due to to the limited number of patients characterized by these molecular lesions, no clinical effect was evident for

the presence of *ASXL1* or *DNMT3A* as single mutation. Unfortunately, no *IDH2* alterations were identified in our cohort, but this mutation is not frequently altered in MF, accounting for about 2% of the patients. (16) In addition, our analysis revealed that NRM was negatively influenced by MIPPS70-plus high and very high risk class as well as the presence of a *TP53* mutation. On the other hand, relapse rate was not associated with any of the molecular features taken in consideration but rather by the burden of the disease. This observation can suggest that high VAF of *JAK2*V617F mutation can be associated to a disease resulting more difficult to eradicate. These results are in contrast with data previously published by Kroger (17) demonstrating that *ASXL1* mutations are associated with higher relapse risk. Moreover, analysis by Tamari and colleagues (14) on the impact of VAF of the most frequent mutations affecting MF did not demonstrate an impact on survival or relapse. These discordances can be due to the different treatment administered for conditioning and to the limited number of patients considered in these studies. We are planning to evaluate a prospective MF patients cohort underwent to HSCT to validate results obtained in a retrospective manner.

To the best of our knowledge, this is the first study in which the presence of individual mutations and, in some case their VAF, can influence the engraftment. The mechanism by which the presence of these mutations mediated the likelihood of engraftment success need to be elucidated by further biologic evaluations.

In conclusion, our data demonstrated that the application of molecular based scoring systems is crucial for an appropriate indication to transplant for MF patients with an adequate timing. Indeed, transplantation can lead

to a significant cure rate of MF patients no matter the presence of HMR mutations.

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CHAPTER 5

CONCLUSIONS AND FUTURE PERSPECTIVE

5.1 Conclusions and future perspectives

Myeloid malignancies are a heterogeneous group of diseases characterized by a common myeloid clonal origin. As deeply described in this work, current diagnosis of myeloid malignancies has rapidly evolved thanks to the introduction of NGS in specialized hematology laboratories which developed and applied to clinical research by gene panels for targeted sequencing. The great efforts in onco-hematology research and the technological advances of the last decades have greatly increased our understanding of the molecular landscape of myeloid disorders and their prognosis as well as their different responses to treatments. However, in every single group of these neoplasms the clinical course could be heterogeneous and not easily predictable and, thus, it is necessary to invest other resources to deepen the understanding of these diseases. Moreover, the great challenge characterizing research in onco-hematology is translating the results obtained from biological studies into clinically relevant and less and less toxic therapeutic strategies.

Data obtained by our group and described in papers included in this work contributed to a better molecular characterization of AML and MF and to help managing of the patients affected by these diseases.

In chapter 2, we reported our findings obtained by sequencing a cohort of AML patients enrolled in a randomized clinical trial. Our data confirmed that the identification of *CEBPA*, *NPM1*, and *FLT3-ITD* mutations, alone or in combination, remains crucial to define patient subgroups with different prognoses.

Particularly, the presence of a double mutation in *CEBPA* gene was known to be associated with a subgroup of patient characterized by a particularly favorable outcome. The systematic application of the NGS technology is extremely helpful not only to speed up the diagnostic procedure but also allowed the identification of a mutation affecting *CEBPA* gene in the basic leucine zipper domain (bZIP). This mutation is now considered the most important factor to predict a favorable outcome rather than the *CEBPA* bi-allelic mutational status of CBPA (1). In light of this, we are re-analyzing our *CEBPA* mutations from the cohort of patients studied by NGS not only within the group of patients enrolled into the NILG 02/06 clinical trial but also all the others routinely followed-up at our hospital.

On the contrary, *FLT3*-ITD proved as the most relevant marker of unfavorable prognosis no matter the co-presence of *NPM1* alteration in our NILG cohort of patients which were treated in pre-*FLT3* inhibitors era. We confirmed a gradient effect played by *FLT3*-ITD allele burden on survival since, the *FLT3*-ITD with a low allele burden was still associated to a negative outcome. Our findings were in line with other previous reports (2,3). Data published by different groups, included ours, prompted a revision of risk classification by ELN. Indeed, ELN released in 2022 indicate that AML with *FLT3*-ITD with no other additional adverse-risk genetic alterations are now categorized in the intermediate-risk group, irrespective of the allelic ratio or concurrent presence of *NPM1* mutation.

In chapter 3 we investigated the clinical significance of the presence of chromatine-splicesome (CS) mutations in a large cohort of newly diagnosed AML patients enrolled into the NILG trial. We identified a subgroup of

patients characterized by a clinical outcome similar to AML secondary to a previous myelodisplastic syndrome or myeloproliferative disorders. In this study, we demonstrated that patients carrying at least one variant allele of *ASXL1*, *STAG2*, *BCOR*, *EZH2*, *PHF6*, *SRSF2*, *SF3B1*, *U2AF1*, *ZRSR2*, *RUNX1* and *KMT2A* showed clinical characteristics closer to sAML, in terms of both clinical-biological characteristics and outcomes. Indeed, the CS-AML group showed a markedly worse 5-years OS and DFS compared to *de novo* AML. We have pointed out that CS-AML more closely resembles sAML than *de novo* AML, even in the absence of myelodysplasia-related cytogenetic abnormalities and this observation is in keeping with other papers in literature (2,4–6). The recently updated ELN recommendations took into account these findings from different research groups and consider a new adverse risk category the AML harboring a mutation in at least one of the *myelodysplasia-related genes (ASXL1, BCOR, EZH2, RUNX1, SF3B1, SRSF2, STAG2, U2AF1*, *or ZRSR2*), overcoming the previous category defined only on the basis of *ASXL1* or *RUNX1* gene mutation. In addition, CPX-351, a dual-drug that encapsulates cytarabine/daunorubicin, is now labeled as the treatment of choice induction therapy for secondary AML as well as for AML with myelodysplasia-related genetic abnormalities, since it was demonstrated to improve significantly the overall survival compared to conventional 7+3 chemotherapy in this category of patients (7).

Despite the huge advance made in genetics characterization of AML, standard karyotype may technically fail or may miss to identify abnormalities for the limited number of metaphases taken into consideration. Moreover, few patients (about 2% in our cohort) resulted

normal for conventional cytogenetics and for molecular alterations affecting the myeloid neoplasm associated genes. Third generation sequencing was developed to yield long-read genome sequencing and thus to reveal structural abnormalities that might escape conventional karyotype analysis. Moreover, sequencing of a wider genome region, including regulatory and intronic sequences might identify rarer AML related genetic abnormalities and provide useful information. In this context, a group of colleagues from Florence is conducting a study aimed to detect, by Nanopore sequencing, the loss or acquisition of genetic material in normal karyotype AML. Preliminary evidences have suggested that these additional mutational profile may be associated with an adverse clinical outcome. (8) Since an independent validation cohort was required to validate their results, we are currently sequencing by Nanopore samples from our AML patients randomized in the conventional treatment arm and described in the NILG study of chapter 2. Database including both sequencing data (next or second and third generation) and clinical characteristics is in preparation for statistical analysis.

In chapter 4, we evaluated the post-transplant outcome of MF patients enrolled into a multicentric GITMO perspective clinical trial. These patients were classified according to the different scoring systems so far available for this disease including MIPSS70 scoring systems (9,10) which also consider the prognostic value of HMR mutations. Target gene sequencing allowed us to classify patients included in the trial by MIPSS scoring systems obtaining that the great part of them resulted high or very high risk patients. The presence and the number of mutations defined as

high risk mutations found in MF patients, as well as MIPSS risk classification did not impact on OS or PFS in this cohort of patients who underwent allo-HCT. These results suggest that the transplantation may overcome the poor prognosis associated to these clinical and molecular features. However, some of the characteristics analyzed in our study, i.e. the presence of individual mutation, as well as allele burden of *JAK2*-V617F, seemed to affect non relapse mortality, probability to relapse and engraftment. Nevertheless, we have to take into account the limited number of patients considered in this investigation. Thus, we are analyzing a prospective cohort of MF patients underwent transplant and routinely referred to our center, considering the post-transplant outcome, but also the MRD status evaluating sample collected for chimerism assessment. MRD evaluation will be performed by digital droplet PCR (ddPCR) monitoring the mutations (SNV or small insertion and deletions) identified before transplant. The optimization of the ddPCR set up has been concluded and the MRD analysis is starting.

In conclusion, over the last three years we systematically applied the high throughput sequencing of myeloid neoplasms. This led to a significant improvement of our knowledge of the biology of these diseases and led to change the diagnostic work-up of these patients and throughout their clinical course, transplant included. NGS in the clinical laboratory has evolved and will continue to evolve over time adapting to increasing clinical needs and providing ideal throughput per run performable quickly and cost-effectively. These advances in technology will provide a huge number of information that will need to be managed and integrated to clinical features,

also in consideration of the wide offer of target drugs that result adequate only to a restricted category of patients.

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