Impact of co-mutations and targeted therapy in NPM1 and FLT3 mutated acute myeloid leukemia

Guadalupe Oñate Hospital

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IMPACT OF CO-MUTATIONS AND TARGETED THERAPY IN *NPM1* AND *FLT3* MUTATED ACUTE MYELOID LEUKEMIA

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Doctoral thesis / 2023

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A Lucía y Àlex,

por recordarme cada día qué es lo realmente importante

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ABBREVIATIONS

2-yr	2 year
5-yr	5 year
AlloHCT	Allogeneic hematopoietic stem cell transplant
AML	Acute myeloid leukemia
AML/MDS	Acute myeloid leukemia/myelodysplastic syndrome
ARA-C	Cytarabine
Aza	Azacitidine
CETLAM	Spanish cooperative group for the study and treatment of AML
CH	Clonal hematopoiesis
CHIP	Clonal hematopoiesis of indeterminate potential
CI	Confidence interval
CIR	Cumulative incidence of relapse
CR	Complete remission
CR1	First complete remission
CR2	Second complete remission
CT	Chemotherapy
DIC	Disseminated intravascular coagulation
DNMT3A	DNA (cytosine-5)-methyltransferase 3A
DNMT3Amut	mutated DNMT3A
DNMT3Awt	wild-type DNMT3A
EFS	Event-free survival
ELN	European LeukemiaNet
ELN-17	ELN 2017 AML classification
ELN-22	ELN 2022 AML classification
FAB	French-American-British classification
FDA	Food and Drug Administration
FLT3	FMS-like tyrosine kinase 3
FLT3high	FLT3-ITD high allelic ratio
FLT3low	FLT3-ITD low allelic ratio
FLT3wt	wild-type FLT3

HiDAC	High-dose cytarabine
HMA	Hypomethylating agents
ICC	International consensus classification
ITD	Internal tandem duplication
LDAC	Low-dose cytarabine
LFS	Leukemia-free survival
MFC	Multiparametric flow cytometry
molLFS	molecular leukemia-free survival
MRD	Measurable residual disease
NGS	Next-generation sequencing
NPM1	Nucleophosmin gene
NPM1mut	mutated NPM1
NPM1wt	wild-type NPM1
ORR	Overall response rate
OS	Overall survival
PCR	Polymerase chain reaction
R/R	Relapsed/refractory
RAS	Rat sarcoma
RR	Risk of relapse
TKD	Tyrosine kinase domain
TKI	Tyrosine kinase inhibitors
TRM	Therapy related mortality
WBC	White blood cell count
WHO	World health organization

CONTENT

ABSTRACT

Acute myeloid leukemia (AML) is a malignant neoplasm of the bone marrow with a complex genetic landscape and variant clinical outcome. The recent advances in molecular diagnostic techniques and targeted therapies have revolutionized the field, but much is to be improved in the interpretation of co-mutational patterns and clonal evolution, its integration in risk classifications, and its response to targeted therapy. The main goal of the research project presented here is to identify relevant prognostic markers to accurately predict the outcome of patients with AML, and to guide clinicians toward the best therapeutic strategy. We specifically focused on patients that harbored *NPM1* and *FLT3* mutations and how the presence of *DNMT3A* co-mutation influenced their outcome. We found that *DNMT3A* mutations did not modify the prognosis of AML patients with mutated *NPM1* considered favorable by the ELN-17 classification, although closer MRD monitoring is recommended to detect a molecular relapse, and guide a preemptive treatment strategy. We also identified a particular adverse group among patients with the triple association of *NPM1*mut/*DNMT3A*mut and *FLT3*-ITD high allelic ratio. On the other hand, we investigated how the incorporation of FLT3 inhibitors has modified the long-established poor outcomes of patients with AML and *FLT3* mutations in a large cohort of homogeneously treated patients, and we found that midostaurin improved the prognosis of all the *NPM1*mut/*FLT3*mut molecular subsets.

RESUMEN

La leucemia mieloide aguda (LMA) es una neoplasia hematológica originada en la médula ósea con un complejo panorama mutacional y un pronóstico altamente variable entre pacientes. En los últimos años, la combinación de avances en estudios moleculares y el desarrollo de terapias dirigidas han revolucionado esta patología. Sin embargo, quedan muchas incógnitas por resolver, especialmente en relación al impacto de patrones de co-mutación y evolución clonal, su integración en las escalas pronósticas y sus variaciones de respuesta al uso de terapia dirigida. El principal objetivo del presente proyecto de investigación es identificar factores pronósticos que permitan predecir de manera precisa la evolución y el pronóstico de loa pacientes con LMA, y a su vez orienten al clínico en la toma de decisiones terapéuticas. En concreto, nos hemos centrado en el estudio de pacientes con LMA que presentan mutaciones de *NPM1* o *FLT3*, y en como la presencia de mutaciones en *DNMT3A* altera su pronóstico. Hemos evidenciado que las mutaciones de *DNMT3A* no modifican la respuesta y supervivencia de pacientes con mutación de *NPM1* catalogados como favorables según la clasificación ELN-17. En estos casos, sin embargo, es recomendable realizar un seguimiento muy estrecho de la enfermedad residual para poder detectar las recaídas moleculares y así iniciar tratamiento previo a la recaída morfológica. Además, hemos identificado como un grupo de pronóstico especialmente adverso a aquellos pacientes con triple co-mutación de *NPM1/DNMT3A* y *FLT3*-ITD con ratio alélica elevada. Por otro lado, hemos analizado como la incorporación de los inhibidores de FLT3 han modificado el pronóstico adverso largamente establecido de los pacientes con LMA y mutaciones de *FLT3*. Para ello, analizamos los resultados de una cohorte prospectiva tratada homogéneamente antes y después de la incorporación de midostaurina, y observamos que la adición de este agente ha mejorado la supervivencia y disminuido las recaídas de los pacientes con *NPM1*mut/*FLT3*mut en todos los subgrupos moleculares.

1. INTRODUCTION

1.1. Acute myeloid leukemia

1.1.1. Definition and cellular origin

Acute myeloid leukemia (AML) is a malignant neoplasm of the bone marrow characterized by the clonal expansion and differentiation arrest of myeloid progenitor cells resulting in impaired hematopoiesis and marrow failure.(1) It is a phenotypically and genetically heterogeneous disease, with variant clinical outcome.(2-5)

AML may appear *de novo* or as a secondary disease during the evolution of a pre-existing hematological neoplasm, mainly a myelodysplastic syndrome or myeloproliferative disease. It can also develop as a result of DNA damage induced by prior exposure to cytotoxic agents, for example for the treatment of a previous unrelated neoplasm.

Similar to other myeloid malignancies, AML arises from the acquisition of somatic mutations in hematopoietic stem (progenitor) cells. Results from large comparative genomic studies that analyzed matched tumor-normal samples showed that AML involves a lower number of coding mutations compared to other human cancers.(6) However, the clinical and prognostic heterogeneity between AML patients underlies a high genetic combinatorial diversity and involvement of other elements apart from gene mutations, such as changes in gene expression(7-9), methylation profiles and modulation of the bone marrow microenvironment. (10)

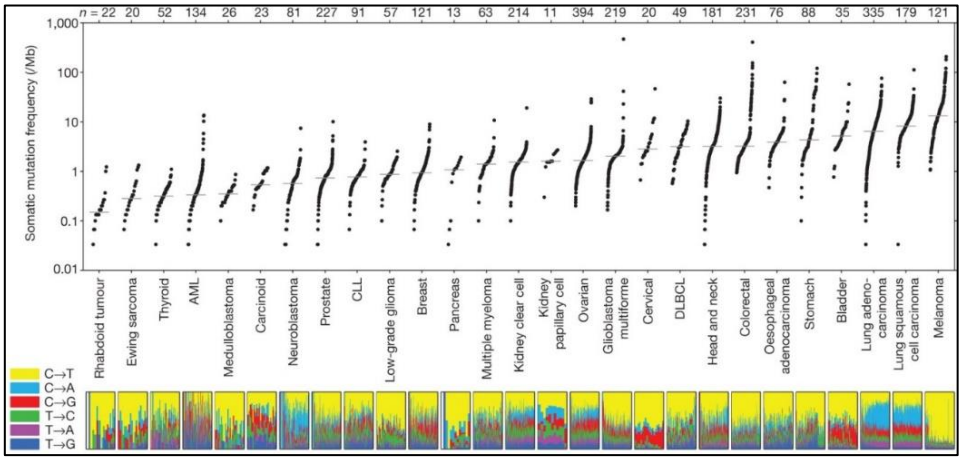


Figure from Lawrence et al. (Nature 2013): Somatic mutation frequencies observed in exomes from 3.083 tumor–normal pairs. Tumor types are ordered by their median somatic mutation frequency, with the lowest frequencies on the left.

1.1.2. Clonal hematopoiesis

Clonal hematopoiesis (CH) results from an expansion of cells that harbor an initiating driver mutation with or without an accompanying cytopenia. Several studies suggest that CH might be a consequence of the aging hematopoietic system.(11, 12) Approximately 2% of persons older than 75 years old present with somatic events in their bone marrow cells such as genomic insertions and deletions (indels) or loss of heterogeneity accompanied by normal or near normal blood counts. This phenomenon is known as clonal hematopoiesis of indeterminate potential (CHIP) which, per se, exhibits low oncogenic potential; whereas in some of these individuals a myeloid neoplasm develops in the follow-up, others will never develop an overt myeloid disorder during their lifetime. (13)

In a NEJM study by Jaiswal et al.(14) 17.182 persons of age 19 to 108 without a known active hematological condition were analyzed to detect acquired single nucleotide variants and small indels through whole exome sequencing of peripheral blood samples. In this study, mutations implicated in hematological cancers were very rare in individuals <40 years, but its frequency progressively increased with age, from 5.6% in persons 60-69 years, 9.5% in 70-79 years, 11.7% 80-89 years and 18.4% in persons above 90 years.

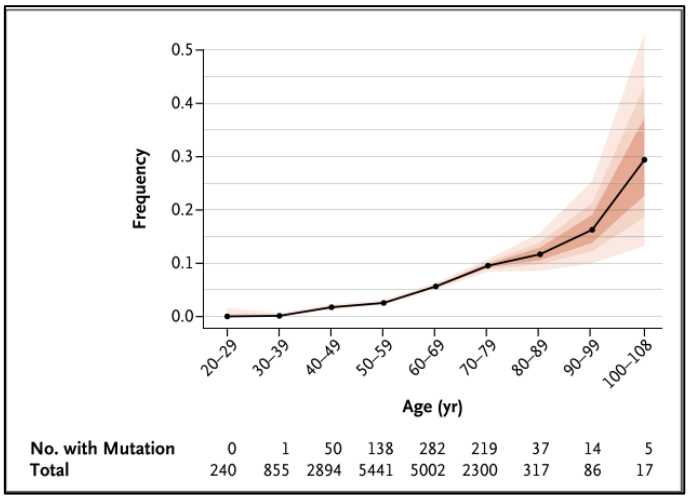


Figure by Jaiwal et al. (NEJM 2014). Prevalence of somatic mutations according to age.

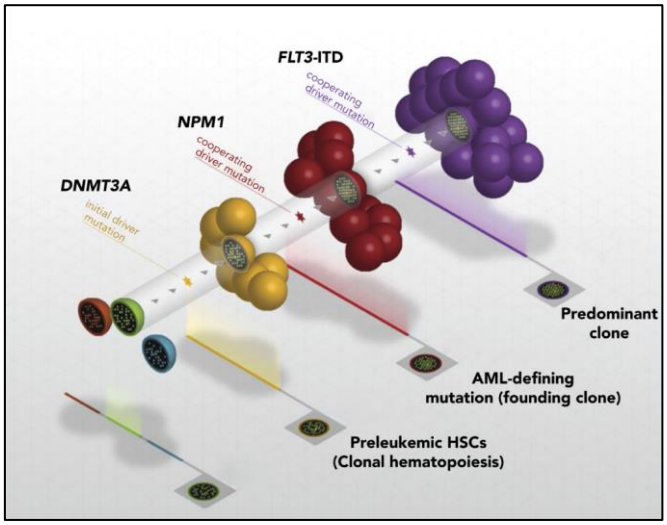
The most frequent mutations associated with CHIP are found in the genes *DNMT3A*, *ASXL1* and *TET2* (also known as DAT genes) and have been related with an increased risk of developing an hematological cancer, although they do not have the potential to initiate a leukemia.(14, 15) These preleukemic hematopoietic stem cells harboring only

the initial driver mutation have also been found in the bone marrow of patients with an AML in remission.(16, 17)

Finally, individuals with CHIP, especially with *DNMT3A* or *TET2* mutations may be at relatively higher risk of developing progressive atherosclerosis and consequent cardiovascular disorders, due to inflammatory disorders related to these mutations.(18, 19)

1.1.3. The two-hit hypothesis and clonal evolution

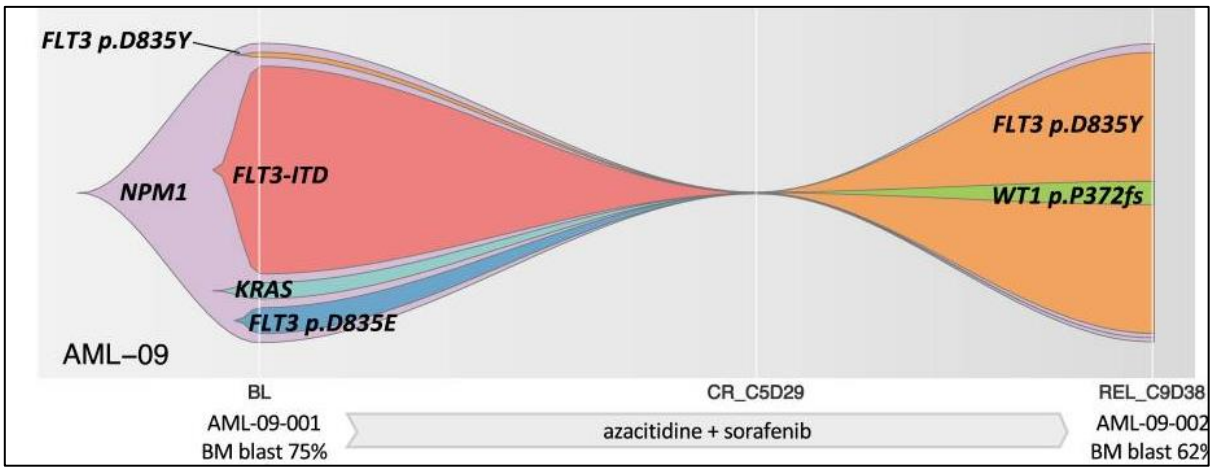
Since the publication of the first AML genome (20), multiple studies have identified recurrent somatic gene lesions involved in the pathogenesis of AML which proved that AML is a complex and dynamic disease.(21, 22) The double hit hypothesis suggests that AML arises due to the acquisition of at least two mutations, one that confers a proliferative advantage (Class I mutations), and one that impairs differentiation (Class II mutations). Mutations in genes involved in epigenetic regulation (*TET2, IDH1, IDH2, ASXL1, DNMT3A)* frequently occur as early founder events in preleukemic progenitor cells before leukemogenic events such as mutations in *NPM1* or signaling molecules.(23)



Example of mutation acquisition in *NPM1* mutated AML (from Falini et al. Blood 2020)

Recently, single-cell genomic analyses have revealed different patterns of mutational co-occurrence at the cellular level. These studies enabled for the reconstruction of the clonal architecture and mutational evolution of AML since the diagnosis to the relapse and in response to treatment, specially targeted therapy.(24, 25)

Thus, two major clonal evolution patterns have been identified in AML: one in which the founding clone gains mutations and evolves into the relapse clone, and another in which a subclone of the founding clone survives initial therapy, gains additional mutations and expands at relapse. Overall, AML relapses result from the incomplete eradication of the leukemic founder clones rather than the emergence of unrelated novel clones. (26-28)



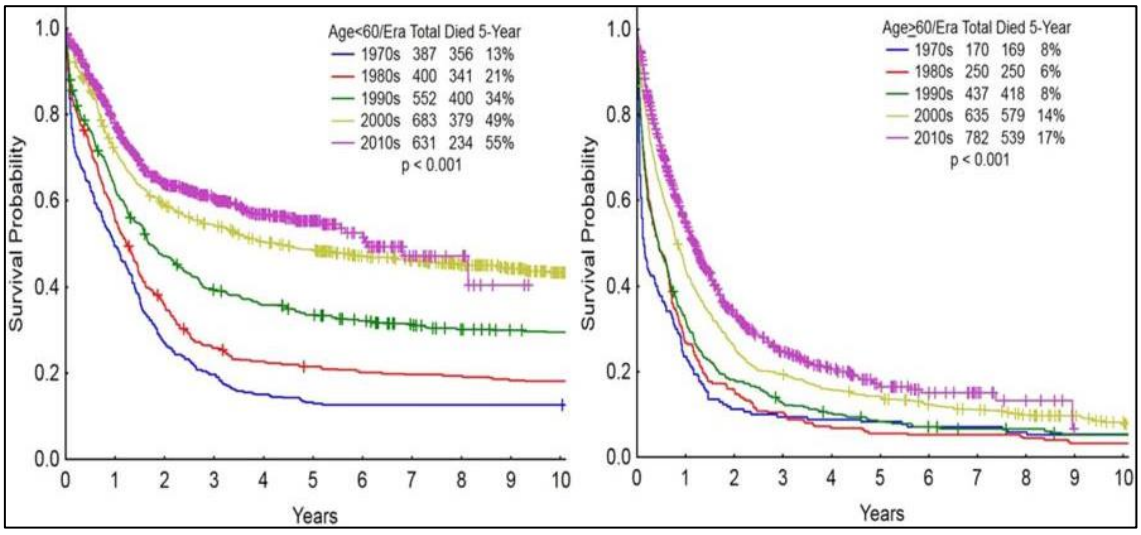
From Morita et al. (Nat Commun 2020): inferred clonal evolution pattern of an AML patient from diagnose to relapse based on the single-cell genotype data

1.1.4. Demographics

There are approximately 18.000 new cases of AML diagnosed in Europe every year representing 0.6% of all cancers.(29) In adults, AML represents the 5th neoplasm in frequency and it is the most predominant acute leukemia, representing 85% of all adult acute leukemias in contrast to 15% of the leukemias in children. Males are 1.2-1.6 times more likely to develop AML than females, this difference accentuated with age.(30-33)

AML characteristically affects older adults with a median age at diagnosis of 64-67 years. The overall annual crude incidence of AML is 3.7-4.3 cases/100.000 person-years but varies throughout age groups with an estimated age-adjusted AML incidence rates for those ≥65 years of 20.1 cases/100.000 person-years while in <65 years is 2 cases /100.000 person-years.(34-36)

Survival of AML patients also differs according to age. Thus, in North American records, the 5 years (5-yr) overall survival in AML patients <50 years is 63% while in patients between 50 and 64 years is 38% and in patients >65 years is 10%.(37, 38)



From Kantarjian et al. (Blood Cancer J 2021) Survival of de novo AML patients at MD Anderson (1970–2017) by age and treatment era

1.1.5. Clinical manifestations

AML typically presents with a rapid onset of symptoms and may be fatal within weeks or months when left untreated. The clinical symptoms in AML patients result from the proliferation of abnormal blasts in the bone marrow, which interfere with normal hematopoiesis and cause bone marrow insufficiency and pancytopenia. This results in symptoms of pallor, weakness and easy fatigability, infections of variable severity and/or hemorrhagic events.(39) AML can also associate with life-threatening situations such as leukostasis, coagulopathy or tumor lysis syndrome.

Bone marrow insufficiency

The decreased production and maturation blockade of normal hematopoietic cells result in:

- Anemia and associated pallor, fatigue, hypoxia, heart failure, respiratory failure
- Thrombocytopenia and hemorrhage that can range from mild mucosal bleeding to severe hemorrhagic events such as central nervous system or pulmonary bleeding.
- Deep neutropenia associated to high-risk bacterial and fungal infections, sepsis and septic shock.

Extramedullary infiltrations

Any tissue can be virtually invaded by leukemic cells, although the most frequently affected sites are skin, bone, periosteum, and lymph nodes.(40) Central nervous system involvement is rare. Certain subtypes of AML are more commonly associated with skin infiltration and the most frequent association occurs with acute myelomonocytic and monocytic differentiation, both typically associated with mutated *NPM1*, with skin involvement in up to 50% of patients.(41) Extramedullary disease may present simultaneously or precede bone marrow disease, and may be seen in relapse.

Hyperleukocytosis and leukostasis

Up to 18% of patients with AML present with a white blood cell count (WBC) greater than 100.000/µL.(42) Monocytic and myelomonocytic AML subtypes as defined by the French-American-British (FAB) classification and patients with *FLT3*-ITD mutations or abnormalities involving the MLL gene on chromosome 11q23 have been identified as risk factors for hyperleukocytosis.(43-45)

Leukostasis is a phenomenon that leads to diffuse cerebral and pulmonary microcirculatory failure from the sludging of leukemic blasts into capillary vessels. Symptoms may arise from the involvement of any organ system but intraparenchymal brain hemorrhages and respiratory failure account for the majority of early deaths.(46)

The extent of hyperleukocytosis in AML does not necessarily correlate with the likelihood of developing leukostasis, which suggests involvement of specific molecular interactions between AML blasts and endothelial cells, increased blood viscosity as well as reduced deformability of myeloid blasts compared to both lymphoid blasts and mature myeloid cells.(47)

Tumor lysis syndrome

Tumor lysis syndrome in AML appears due to the extremely rapid cell turnover of leukemic blasts leading to electrolyte imbalances and increased serum levels of uric acid that can culminate in renal failure and fatal cardiac arrhythmias. Treatment entails supportive management of electrolytes, intravenous fluids to maintain urine output, and allopurinol or rasburicase to reduce the production of uric acid.(48-50)

Coagulopathy

Disseminated intravascular coagulation (DIC) has been reported in up to one third of patients with non-promyelocytic AML. An elevated leukocyte count is a strong risk factor for the development of DIC. It is characterized by both excess activation of the coagulation system due to the release of tissue factor from endothelial cells and leukemic cells and increased fibrinolysis that leads to hemorrhagic and thrombotic events.(51)

1.2. Diagnosis of AML

1.2.1. Morphology and evolution of diagnostic classifications

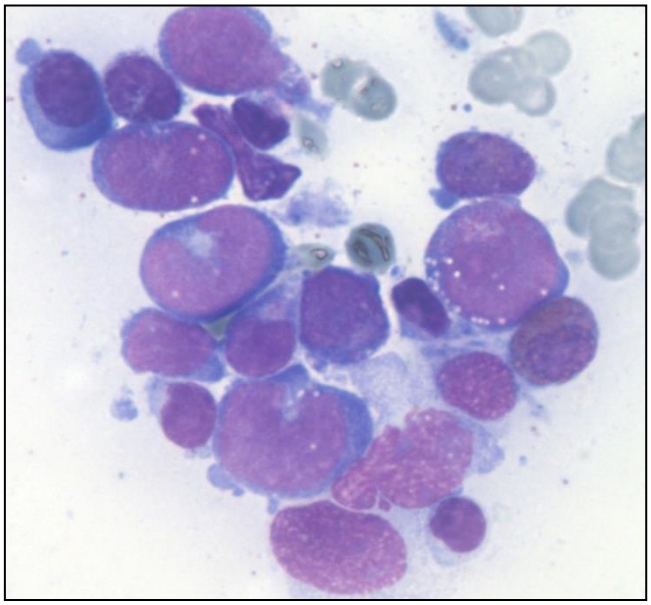
Morphological evaluation of both peripheral and marrow smears is the first step for the diagnosis of many hematological malignancies. Since Ehrlich's revolutionary discovery of the benefits of blood cell staining with aniline dyes in 1877 (52), the morphological description of blood smears was a first approach for physicians to understand the cause of the clinical disparities they perceived in patients.

The correlation between patients with similar clinical characteristics and specific morphological traits in their bone marrow lead to the elaboration of the first classification of myeloid neoplasms in 1967. This was performed by a group of morphologists from France, the United States, and Great Britain (also known as FAB) who suggested a classification system designed to standardize the different morphological types of AML.(53) The FAB classification remained reference in the field until the late 90s, when the first World Health Organization (WHO) classification of myeloid neoplasms was published.(54, 55) In these classifications, the definition of acute leukemia was established when either ≥30% (FAB) or ≥20% (WHO) blasts were observed in the bone marrow or the peripheral blood, and was further classified according to defining morphology traits and histochemistries of the blast cells that determined lineage (ie: monocytic, erythroid) and maturity.

Recently the standard cut-off of ≥20% blasts for the diagnosis of AML is being revisited since, based on data from various studies, some authors. debate that myeloid malignancies are a continuum specially in the presence of recurrent genetic alterations.(56) Thus, the recently published international consensus classification (ICC 2022) considers patients with myeloid dysplasia and >10% blasts as acute myeloid leukemia/myelodysplastic syndrome (AML/MDS) in order to enable access of these patients for AML trials and therapies.(57)

The advances in diagnostic techniques and translational research were reflected in the latest versions of AML classifications that progressively increased cytogenetic and molecular criteria to the detriment of morphological traits.(1, 57-59) However, morphology is still essential in various settings. For instance, to raise suspicion of the diagnosis in the emergency department and allow a promptly start of support measures

and referral of patients to tertiary hospitals if necessary. It is also of utmost importance to rapidly identify morphological features of specific leukemia subsets such as Auer rods in acute promyelocytic leukemia, an entity highly associated with potentially fatal coagulopathy that requires early initiation of directed therapy with retinoids. Finally, it may guide clinicians towards a specific acute leukemia lineage (ie: lymphoblastic vs myeloid) or even raise suspicion of some genomic entities (ie: eosinophilia and inv(16), monoblastic with indentation and *NPM1* mutation) while waiting for more specific techniques. Finally, it must be taken into account that although nowadays most leukemia centers have access to the newest technologies for diagnosis and follow-up, the global consensus criteria for response assessment after therapy still defines complete response (CR) as the absence of morphological disease (<5% blasts in bone marrow evaluation).(60)



Picture from Oñate et al. Med Clinica 2019 (61). MO. x40 MGG staining in AML with inv(16)(p13q22)

1.2.2. Multiparametric flow cytometry (MFC)

Normal maturation and differentiation of the diverse hematopoietic cell lineages is associated with sequential antigen expression patterns. Neoplastic blasts usually harbor an aberrant antigen expression that allows distinction from normal immature cells, such as overexpression or lack of certain antigens or detection of markers usually not present on cells of that particular lineage.(62)

MFC is required at diagnosis to stablish the lineage of acute leukemia patients and identify potential targets (ie: CD33). MFC is also essential for the monitoring of measurable residual disease (MRD). There are two main strategies to perform flow MRD: either following the antigen aberrations present in leukemic blasts at diagnosis (also known as "leukemia-associated immunophenotype" or LAIP) such as lymphoid markers in myeloid blasts, CD11b in immature CD34 cells, or over expression of CD33; or by the identification of the tumoral cells compartment in the histograms that is usually empty in healthy individuals (also known as the "different-from-normal" approach). (63)

Multiple studies have validated the value of the prognostic impact of flow MRD in AML (64-68), however some of its limitations are the lack of tumor specific antigens, changes in antigen expression of the relapsed clone, the difficulty of interpretating results that requires an experienced analyst and the variability of the technique between centers.

1.2.3. Genetic studies

Genomic analysis should be performed at diagnosis and at the relapse/refractory setting for optimal disease characterization and treatment choice.(69) Genetic diagnosis in AML starts with cytogenetics (chromosome Giemsa banding and/or fluorescent in situ hybridation) to identify chromosomal abnormalities and recurrent translocations. These techniques identify chromosomal abnormalities in 55% of adult AML patients. However, normal karyotype constitutes the single largest cytogenetic group of AMLs, estimated to account for 45% of adults with de novo AML. Molecular techniques are able to reveal recurrent genetic alterations in more than 85% of patients with normal karyotype AML.(7, 8, 70)

One of the first techniques that enabled the study of single-gene mutations was the Sanger method, developed by Frederick Sanger and colleagues in 1976. This is a DNA sequencing method based on the random incorporation of chain-terminating dideoxynucleotides by a DNA polymerase during in vitro DNA replication.(71) Later on, molecular analysis were revolutionized by the discovery of the polymerase chain reaction (PCR) by Dr. Kary B. Mullis (who was awarded Nobel Prize of Chemistry in 1993) that amplifies millions to billions of copies of a specific segment of DNA, allowing its study in greater detail.

Since then, multiple assays based on PCR-Sanger techniques have been progressively incorporated in the diagnostic work-up of AML to study single gene mutations with known impact in the disease, both for risk stratification and/or targeted therapy. However, screening for gene mutations is an evolving field of research; and single-gene analyses are being increasingly replaced by diagnostic panels with multiple genes being amplified at the same time.

Next-generation sequencing (NGS), massively parallel or deep sequencing are related terms that describe a DNA sequencing technology which has revolutionized genomic research. NGS performs sequencing of millions of small fragments of DNA in parallel, and bioinformatics analyses are used to piece together these fragments by mapping the individual reads to the human reference genome. NGS can be used to sequence entire genomes or focalized to specific areas of interest with customized gene panels. (72)

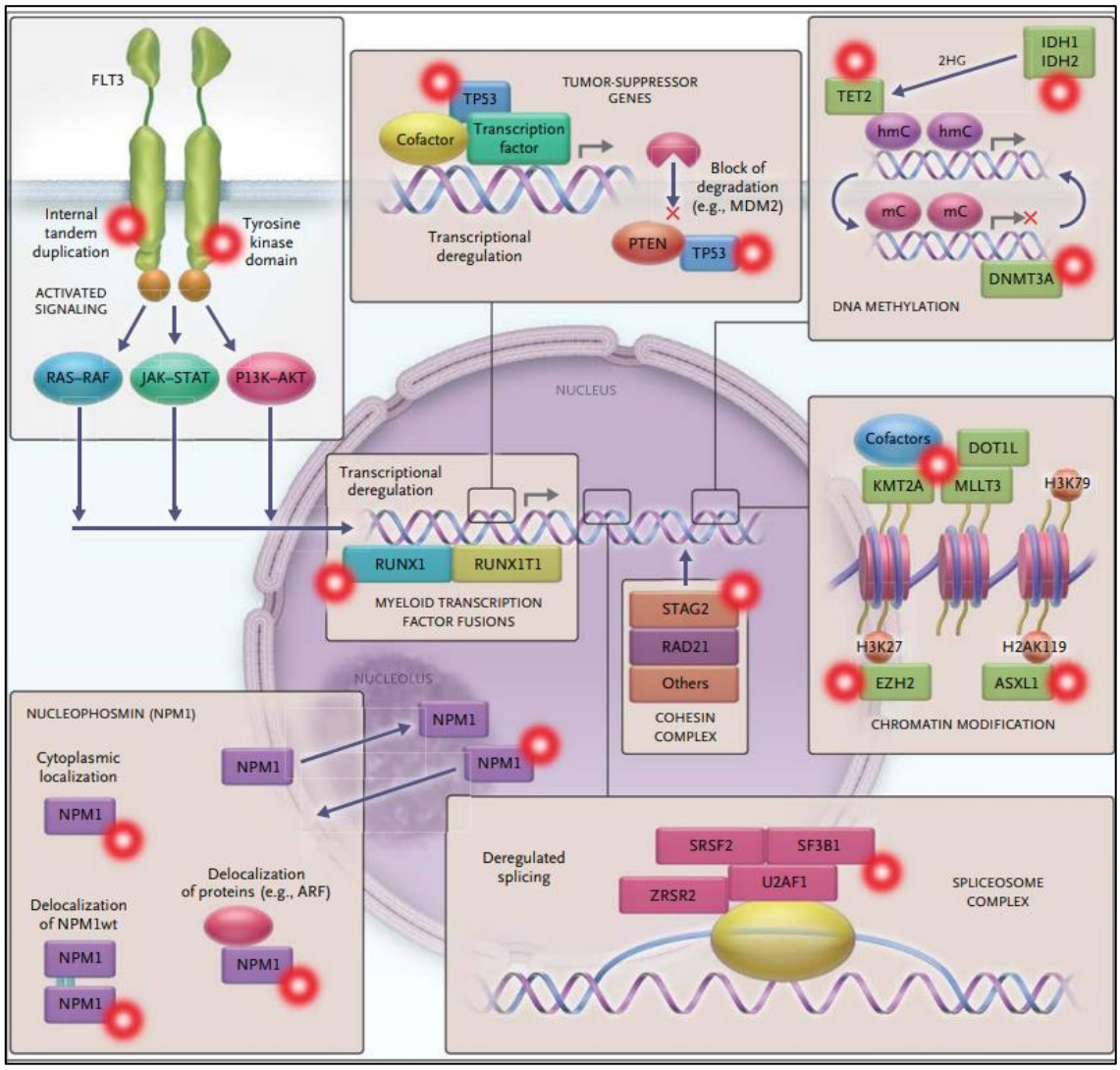
The increasing knowledge of the molecular landscape of AML lead to the elaboration of a genomic classification in 2016 by Papaemmanuil et al. In their study, they combined data from cytogenetics and molecular sequencing of 111 genes of 1540 adult AML patients and developed a genetic-only classification for AML based on statistical models that compartmentalized AML into 11 mutually exclusive subtypes according to patterns of co-mutations.(5)

Although bulk sequencing informs about leukemia biology and prognostication, it cannot distinguish which mutations occur in the same clone(s), accurately measure clonal complexity, or definitively elucidate mutational order. Thus, single-cell sequencing assays are being developed (for now at the investigational level only), to further understand AML pathogenesis.

1.2.4. Most relevant genes with clinical implications in AML

The most commonly affected genes in AML can be arranged into functional categories according to their cellular function and role in leukemogenesis (22) and the probability of their co-occurrence (5). Here is a brief summary of the recurring AML genes listed by their frequency (as reported by The Cancer Genome Atlas data set of AML patients <55 years).(21) The three most relevant genes for the present thesis (*FLT3, NPM1* and *DNMT3A*) are reviewed separately in depth.

- Signaling genes such as kinases (ie: *FLT3, KIT, PTPN11*) or *RAS* family members (*KRAS, NRAS*) who confer a proliferative advantage of leukemic blasts.
- DNA methylation-associated genes (*DNMT3A, IDH1, IDH2*) that alter the transcription of leukemia related genes by deregulating DNA methylation patterns.
- Myeloid transcription factors, which can have gene fusions (ie: t(8;21) or inv(16)/t(16;16)) or mutations (*RUNX1, CEBPA*) that result in altered transcription and impaired hematopoiesis.
- Chromatin-modifying genes (*ASXL1, EZH2* or *KMT2A* fusion) which lead to transcriptional deregulation by affecting chromatin modification processes (ie: methylation of histones).
- Nucleophosmin gene (*NPM1*) a nuclear-cytoplasmatic shuttle protein in which mutations lead to its aberrant cytoplasmatic localization and altered function.
- Tumor-suppressor genes (*TP53, WT1, PHF6*) that cause impaired degradation and transcriptional deregulation.
- Spliceosome-complex genes (*SRSF2, SF3B1, U2AF1, ZRSR2*) that lead to deregulated RNA processing and aberrant splicing patterns.
- Cohesin complex genes (*STAG2, RAD21*) that lead to impaired accurate chromosome segregation and affect transcriptional regulation.



Representation of the most relevant genomic pathways in AML. From Dohner H, Weisdorf DJ and Bloomberg C Acute Myeloid Leukemia (NEJM 2015)

FLT3

The FMS-like tyrosine kinase 3 (*FLT3)* gene on chromosome 13q12 encodes for the FLT3 transmembrane receptor tyrosine kinase. FLT3 is expressed on lineage-restricted myeloid and lymphoid progenitor cells and it is activated by FLT3 ligand. FLT3 comprises five domains: extracellular, juxtamembrane, tyrosine kinase, kinase insertion, and a C-terminal intracellular domain. It is embedded in several signaling pathways responsible for the cell life cycle, from differentiation to apoptosis. Normally, after binding to the FLT3 extracellular domain, the receptor dimerizes and is subsequently phosphorylated, becoming activated. This receptor further activates downstream signaling cascades such as the phosphatidylinositol 3-kinase (PI3K) and rat sarcoma (RAS), leading to hematopoietic cell maturation and proliferation. Soluble FLT3 ligand concentration is usually very low but can increase exponentially in response to aplasia, activating FLT3 only when necessary. AML cells overexpress FLT3, and *FLT3* mutations lead to tyrosine kinase receptor activation by ligand-independent dimerization, resulting in aberrant proliferation of malignant cells.(73-76)

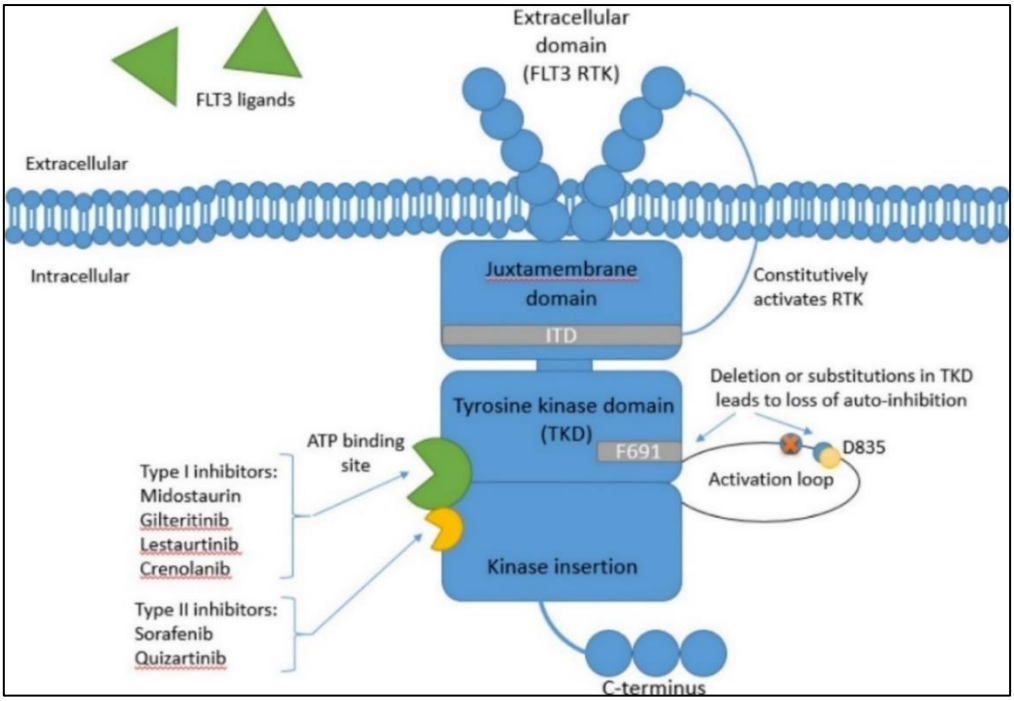
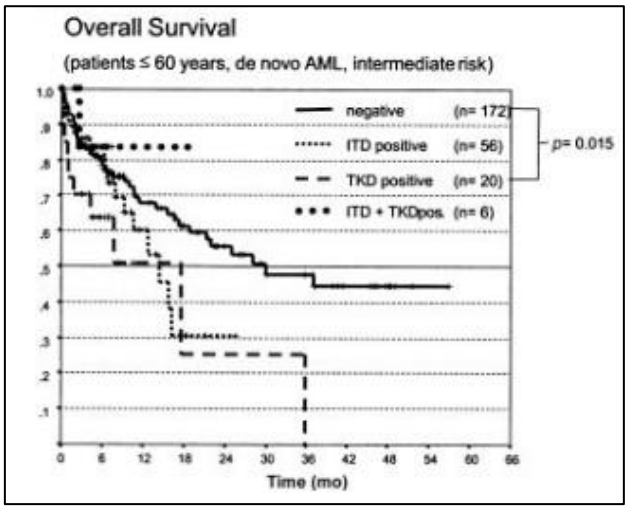


Figure from Zhao et al. A review of FLT3 inhibitors in acute myeloid leukemia (Blood Rev 2023)

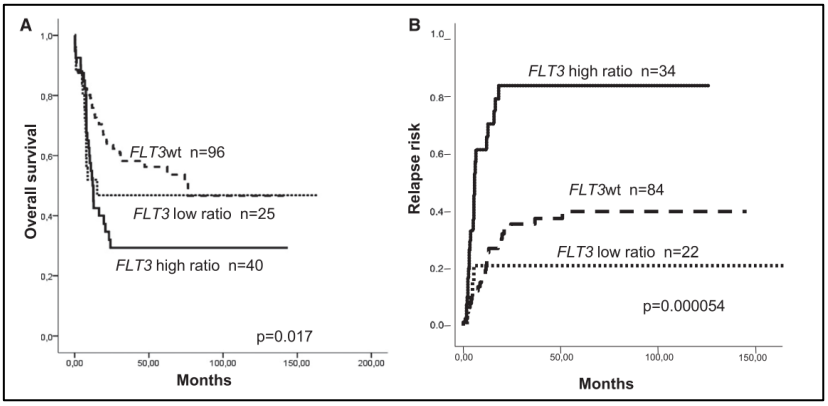
FLT3 is one of the most frequently mutated genes in AML with an incidence of around 30% and is generally associated with a negative outcome.(77) The most frequent mutations of *FLT3* are the internal tandem duplication (*FLT3*-ITD) in the juxta-

membrane domain and point mutations of the TKD2 domain (*FLT3*-TKD), with frequencies of 22% and 8% respectively.(5) Several studies showed that FLT3 mutated patients have an adverse prognosis, with higher relapse rates and worse overall survival than patients lacking these mutations. (43, 78)



Graph from C. Thiede et al (Blood 2002) Overall survival of different types of *FLT3*mut (discontinued lines) and *FLT3* wild-type (continuous line)

The prognosis of patients harboring *FLT3*-ITD depends on several variables, such as the presence of determinant co-mutations like *NPM1,* the allelic ratio of the mutation or the insertion site.(79-83) Regarding allelic ratio, several studies, including the European LeukemiaNet (ELN) 2017 classification emphasized its relevance in risk assessment. (60, 79, 80, 83, 84). In the Spanish CETLAM group, patients are classified according to its ITD/wt allelic ratio as low ratio (<0.5; *FLT3*low) and high ratio (≥0.5; *FLT3*high).



From Pratcorona et al. (Blood 2013). Impact of *FLT3*-ITD allelic ratio on *NPM1*mut-AML

Recently, a study by K. Dohner et al. validating the ELN-17 classification in the RATIFY cohort showed that the cut-off of 0.5 was also the best discriminant value to define patients with different prognosis based on allelic ratio.(81) This differs from the

original RATIFY trial that considered the 0.7 ratio cut-off. However, there is some controversy regarding the specific *FLT3*-ITD allelic threshold that can accurately divide high- and low-risk patients as well as concerns regarding the reproducibility of the technique. These are some of the reasons which led experts to remove the modulating effect of *FLT3*-ITD allelic ratio in the 2022 prognostic classification of the ELN for AML.(69)

NPM1

The nucleophosmin (*NPM1*) gene encodes for an ubiquitous multifunctional shuttling protein with predominant nucleolar localization, with a critical involvement in the nucleolar phase separation and for maintaining the directionality of ribosome biogenesis through the different subcellular compartments.(85, 86) *NPM1* mutations lead to an aberrant cytoplasmatic localization of the protein. These mutations are always heterozygous given that complete loss of *NPM1*wt is embryonically lethal.(87) Most *NPM1* mutations consist of the insertion of 4-base pair in exon 12.

NPM1 is the most commonly mutated gene affecting approximately 30% of adult AML patients.(88) It is considered a recurrent genetic alteration and designates a specific diagnostic category.(1, 57)

According to the ELN-17 recommendations, *NPM1* mutations convey a relatively favorable prognosis only if *FLT3*-ITD is absent or shows a low allelic ratio but, as mentioned previously, there is some controversy regarding the value of *FLT3*-ITD allelic ratio, and the ELN-22 risk-classification considers favorable only *NPM1*mut in the absence of *FLT3*-ITD. Risk stratification according to *FLT3*-ITD allelic ratio requires further validation and it will possibly depend on the treatment setting (ie: FLT3 inhibitors or allogeneic transplant) and co-mutational pattern.

DNMT3A

The DNA (cytosine-5)-methyltransferase 3A (*DNMT3A*) gene is located in the short arm of chromosome 2 and encodes for a DNA methyltransferase which methylates unmodified DNA cytosines residues modulating the expression of several genes.(89, 90) Almost all *DNMT3A* mutations are heterozygous and more than two-thirds cluster at the methyltransferase domain in codon R882, causing loss of methylation activity by disturbing *DNMT3A* tetramerization.(91-95) However, although a precise methylation pattern alteration resulting from mutations in *DNMT3A* has not yet been established (9, 96, 97), a mechanism of leukemogenesis characterized by the upregulation of the hepatic leukemia factor (a specific leukemic transcription factor) has been related to the co-occurrence of *DNMT3A*, *NPM1* and *FLT3* mutations.(98)

DNMT3A is considered one of the founder mutations in AML.(99, 100) It has been associated with age-related clonal hematopoiesis with increasing frequency in normal elderly individuals(101), although there is also evidence of a correlation between *DNMT3A* mutations and *NPM1*mut AML regardless of the age of patients.(102)

Patients with AML and mutated *DNMT3A* (*DNMT3A*mut) are frequently older, and present higher leukocyte and platelet counts compared with wild-type *DNMT3A* (*DNMT3A*wt).(92, 103, 104) *DNMT3A* is the third most frequent mutated gene in AML patients included in intensive chemotherapy trials. It is predominantly observed in AML-*NPM1*mut (73%) and less frequently in patients with mutations in chromatin remodeling genes or genes involved in spliceosome function. Interestingly, a recurrent association of *NPM1*mut/*DNMT3A*mut/*FLT3*-ITD has been observed in 6% AML.(5, 105) The prognostic significance of mutations in *DNMT3A* has been controversial, while some studies found no significant influence in survival outcome others suggest that the co-occurrence of *NPM1*mut/*DNMT3A*mut/*FLT3*-ITD in AML patients is associated with a poorer outcome. (92, 106-108)

1.3. Disease evaluation

1.3.1. Risk stratification

AML is a highly heterogeneous disease with a complex mutational landscape. The incorporation of multiple-gene sequencing platforms to the routine diagnostic work-up of patients has led to major advances in understanding the clinical and prognostic significance of several mutations.

In 1990 a group of experts developed a set of standardized diagnostic and response criteria for AML clinical trials that were reviewed in 2003 to integrate the advances in biology and molecular genetics.(109, 110)

In 2010, an international expert panel on behalf of the European LeukemiaNet (ELN) published recommendations for the diagnosis and treatment of AML.(111) In this report, patients were divided into favorable, intermediate or adverse-risk according to their genetic lesions at diagnosis. Each of these categories show significantly different outcomes regarding response rates and survival as well as therapeutic approaches, most notably with regard to allogeneic hematopoietic stem cell transplant (alloHCT) indication in first complete remission. Since then, two updates (in 2017 and 2022) have been published in order to incorporate the latest advances in AML pathogenesis as well as in diagnostic assays and newer therapies.(60, 69)

In the ELN classifications, the first single-gene mutations that defined risk in patients without definitory cytogenetical abnormalities were *NPM1*, *FLT3* and *CEBPA*. All three mutations have long been considered of prognostic relevance in AML and their presence carries specific prognosis.

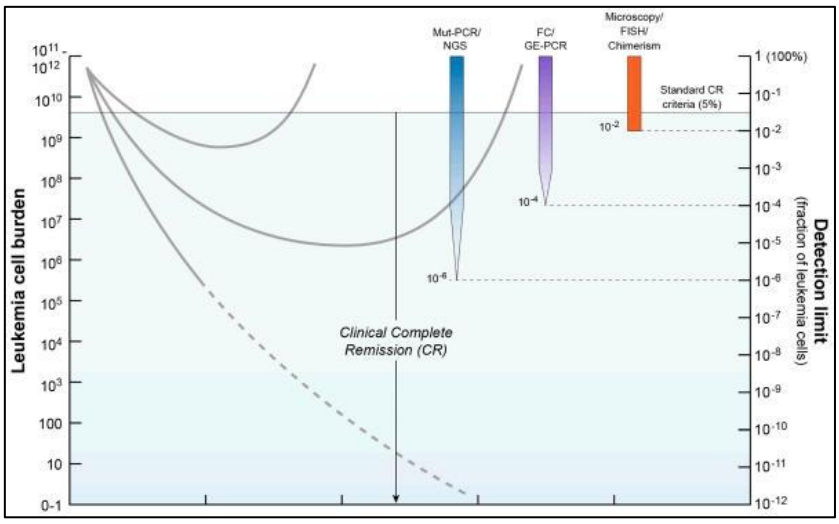
The ELN-17 also included mutations in *TP53*, *RUNX1* and *ASXL1* as part of the adverse category, while the ELN-22 refined some of the previously included genes (b-zip mutations in *CEBPA*, removal of *FLT3*-ITD ratio) and included the presence of dysplasia-related gene mutations (in the absence of co-occurring favorable lesions) as markers of adverse prognosis.

Risk		ELN 2010	ELN 2017	ELN 2022
Favorable		• t(8;21)(q22;q22), *RUNX1::RUNX1T1* • inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB::MYH11* • *NPM1*^{mut} without *FLT3*^{ITD} • Mutated *CEBPA*	• t(8;21)(q22;q22.1), *RUNX1::RUNX1T1* • inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB::MYH11* • *NPM1*^{mut} without *FLT3*^{ITD} or with *FLT3*^{low} • Biallelic *CEBPA*	• t(8;21)(q22;q22.1), RUNX1::RUNX1T1 • inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB::MYH11* • *NPM1*^{mut} without *FLT3*^{ITD} • b-zip in frame mutated *CEBPA*
Intermediate **Normal karyotype and*	I*	• *NPM1*^{mut} and *FLT3*^{ITD} • *NPM1*^{wt} and *FLT3*^{ITD} • *NPM1*^{wt} without *FLT3*^{ITD}	• *NPM1*^{mut} and *FLT3*^{high} • *NPM1*^{wt} without *FLT3*^{ITD} or with *FLT3*^{low} • t(9;11)(p21.3;q23.3), *MLLT3-KMT2A* • Cytogenetic not favorable or adverse	• NPM1^{mut} and FLT3^{ITD} • NPM1^{wt} without FLT3^{ITD} • t(9;11)(p21.3;q23.3), MLLT3-KMT2A • Cytogenetic and/or molecular abnormalities not favorable or adverse
	II	• t(9;11)(p22;q23), *MLLT3-MLL* • Cytogenetic not favorable or adverse		
Adverse		• t(6;9)(p23;q34); *DEK-NUP214* • t(v;11)(v;q23); *MLL* rearranged • -5 or del(5q); -7, abnl(17p); complex karyotype • inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*	• t(6;9)(p23;q34.1); *DEK::NUP214* • t(v;11q23.3); *KMT2A* rearranged • -5 or del(5q); -7, -17/abn(17p) • complex karyotype, monosomal karyotype • inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2,MECOM (EVI1)* • t(9;22)(q34.1;q11.2); *BCR::ABL1* • *NPM1*^{wt} and *FLT3*^{high} • *RUNX1*^{mut} • *ASXL1*^{mut} • *TP53*^{mut}	• t(6;9)(p23;q34.1); *DEK::NUP214* • t(v;11q23.3); *KMT2A* rearranged • -5 or del(5q); -7, -17/abn(17p) • complex karyotype, monosomal karyotype • inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2,MECOM (EVI1)* • t(3q26;v); *MECOM (EVI1)*-rearranged • t(9;22)(q34.1;q11.2); *BCR::ABL1* • Mutated *ASXL1, BCOR, EZH2, RUNX1, SF3B1, SRSF2, STAG2, U2AF1, ZRSR2* • *TP53*^{mut}

1.3.2. Measurable residual disease (MRD)

Achieving a complete remission is the goal of AML treatment. Even though in recent years great advances have been incorporated to AML diagnostic and therapeutical fields, one of the main causes of death for these patients is relapse after having achieved complete remission, either with chemoimmunotherapy or alloHCT. In order to predict which patients will relapse/fail to therapy, genomic prognosticators at diagnosis are well-established as discussed in the previous section. However, risk-stratification is a highly complex process and sometimes not so accurate, since relapses occur in very-good-risk patients and cures are possible in very-poor-prognosis AML. One possible explanation is that risk-stratification at diagnosis does not take into account the contribution of several postdiagnosis factors (ie dosage, compliance, pharmacological resistances) since the only postdiagnosis prognostic factor that is widely accepted is the CR status after the first course of induction therapy.(62)

MRD is defined as the detection of tumoral cells after complete cytological remission through more sensitive techniques. Persistence or reappearance of MRD has an important predictive value for relapse in AML. MRD monitoring is essential in CR patients to indicate treatment modifications and early-interventions to avoid clinical relapse.(112)



From Hourigan C S and Karp JE, MRD in AML (Nat Rev Clin Oncol 2013)

The assays for MRD monitoring have been reviewed in the previous section, multiparametric flow and real-time PCR are the most used techniques with a sensitivity to detect disease of up to 10^{-4} and 10^{-6} respectively.(67) The molecular markers with the best standardization for MRD follow-up are mutations of *NPM1* and chimeric fusion genes (ie: *RUNX1::RUNX1T1 or CBFB::MYH11*).(113, 114)

Although molecular MRD has greater sensitivity, only around 30% of AML patients have a followable genetic marker, thus in 60-70% of AML patients MRD is performed by flow. In some cases, monitoring of the gene overexpression of *WT-1* may be also of help.(115) Newer platforms such as digital PCR or NGS are under development for MRD monitoring but they are not currently stablished in clinical practice. It is important to remember that each technology will need a standardization process to be used in different laboratories, due to the specific technical requirements and sensitivity that must be considered for a correct interpretation of the results.(114, 116, 117)

The prognostic impact of MRD in AML has been repeatedly validated in many studies.(65, 113, 118-125). In 2020, a large systematic meta-analysis from Short et al. analyzed data from eighty-one publications reporting on 11.151 patients and showed a 5-year disease-free survival of 64% for patients with negative MRD vs 25% for those positive, and the estimated overall survival (OS) was 68% vs 34% in the same groups. Impact was validated across all subgroups.(126)

The cut-off to consider negative MRD differs according to the assay. Recently, a panel of international experts on behalf of the ELN elaborated a set of recommendations for MRD assessment.(116) Thus, for MFC 0.1% is the standard cut-off while test negativity by qPCR is defined as cycle threshold ≥40 in at least 2 of 3 replicates, when ≥10 000 copies of the housekeeping gene are measured. Of note, according to the ELN guidelines, molecular relapse is defined as MRD positivity in a previously negative patient, and molecular progression if there is an increase of ≥1 log10 in a previously positive patient.(127) The recommended follow-up timepoints also vary according to the test used, globally the most important landmark is MRD after two cycles of treatment (124), and afterwards MRD assays should be performed every 2-3 months depending on the sample used (peripheral blood or bone marrow) for 2 years following CR.

1.4. Treatment

The goal of treatment in AML varies according to the fitness of the patient. In younger (usually considered <60 years) or in older fit patients who are able to receive intensive chemotherapy (CT) and alloHCT the aim is to eradicate the disease and maintain a sustained complete remission. In unfit patients in which the therapy related mortality (TRM) and the toxicities of intensive therapy surpasses its potential benefit, the goal is to control and possibly transform AML into a chronic disease with reasonable quality of life as long as possible. In any case, the global intent in AML is to achieve a complete response after the first line of therapy, followed by consolidation or maintenance therapy to deepen the remission and maximize response duration.(60, 69, 128-130)

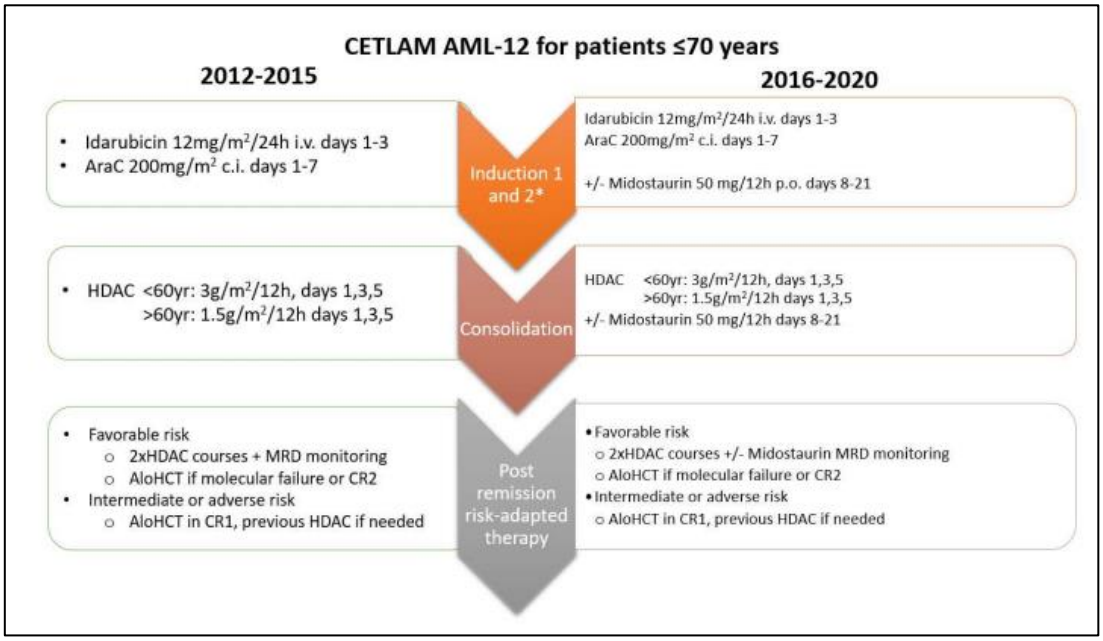
Fitness of patients reflects whether we consider that the patient will tolerate the toxicities of intensive CT regimens and/or alloHCT.(131-134) Age per se is not a criteria of unfitness.(135) It is sometimes a complex decision, especially in patients above 60 years, that requires discussion in specialized committees. In this regard, there have been several studies trying to stablish clarifying criteria based on patient comorbidities and basal status at diagnosis (136, 137), disease characteristics (138, 139) or molecular findings.(140) There are also specific scores to assess eligibility for alloHCT.(141, 142)

Furthermore, considering the prognostic impact of molecular characterization of patients at diagnosis and the increasing availability of targeted therapy, a short delay in starting treatment (if stable) to correctly characterize and identify the best treatment option is nowadays recommended.(143)

1.4.1. Intensive regimens for fit patients

Intensive regimens based on anthracyclines and cytarabine were introduced in 1973 and are still the backbone of most protocols for fit AML patients. The most common induction regimen is based in 1 or 2 cycles of continuous cytarabine during 7 days plus 3 days of daunorubicin commonly known as “7+3”. Following achievement of CR, patients continue with response consolidation therapy either with high-dose cytarabine or stem cell transplant according to the genetic risk at diagnosis and the persistence of MRD after treatment

The decision to perform an alloHCT in first complete remission depends on the risk-benefit ratio and it should be considered when the relapse probability without the procedure is predicted to be >35% to 40%.(144) In many AML treatment protocols, patients of intermediate or adverse risk will be considered for alloHCT in first complete remission, while in favorable patients the decision to transplant would be considered in second or subsequent remission.(69, 112, 145) For instance, in the Spanish cooperative group for the diagnosis and treatment of AML (CETLAM) AML-12 protocol, patients with favorable disease receive 2 to 3 cycles of high dose cytarabine and then proceed to MRD monitoring.



Example of an intensive protocol (AML-12 #NCT04687098) adapted from Spanish CETLAM cooperative group. *Induction-2 only administered in case of partial response

CPX-351 (Vyxeos)

Several trials have been conducted to improve the efficacy and the tolerability of the 7+3 regimen. Vyxeos is a liposomal formulation of cytarabine and daunorubicin at a fixed 5:1 molar ratio, approved for the treatment of adults with newly diagnosed AML with myelodysplasia-related changes or therapy-related AML and has shown reduced mucositis and lower 30- and 60-day mortality, but prolonged neutrophil and platelet recovery time. (146, 147)

1.4.2. Treatment of unfit patients

The classical therapy for unfit AML patients is hypomethylating agents (HMA) azacytidine or decitabine. In monotherapy, they achieve CR rates around 20% and they stabilize the disease in 30% of patients. HMAs are overall very well tolerated and can be given for prolonged periods of time. OS with HMAs at 1 and 2 years is 57% and 35% respectively, which is clearly better than palliative care in this patients (OS 1 and 2 years 16% and 2%).(148, 149)

A number of new agents have been investigated for patients not able to receive intensive therapy. Venetoclax inhibits the BCL-2 (B-cell lymphoma-2) protein that potentiates tumor growth and blocks apoptosis, leading to disease progression and drug resistance. The VIALE trials showed remarkable results with the combination of venetoclax with azacytidine(150) or low-dose cytarabine (LDAC) (151, 152) as primary therapy for unfit patients with untreated AML. In the VIALE-A trial, overall response rate (ORR) was 60-70% and 2-year survival was 40%. Response rate varied depending on the leukemia genomics, with a greater response in *NPM1* or *IDH* mutated patients while mutations of *TP53*, *RUNX1* or *FLT3* were frequently associated with resistance to venetoclax.(153) Venetoclax-based combinations are now widely accepted as the standard first line of treatment for old or unfit AML patients across the world.

In the group of patients with newly diagnosed *IDH1*-mutated AML not eligible for intensive CT, the phase 3 trial comparing the combination of azacytidine with either ivosidenib or placebo observed a median OS in the ivosidenib arm of 24 months vs 7.9 months in the placebo arm and a CR rate of 47% vs 15% in the same groups .(154, 155) Ivosidenib was subsequently approved by the US Food and Drug Administration (FDA) but it is not available in Europe.

Finally, glasdegib is a potent, selective, oral inhibitor of the Hedgehog signaling pathway. In the BRIGHT phase II randomized trial, the addition of glasdegib to LDAC demonstrated superior OS versus LDAC alone.(156) A long-term analysis of the trial showed that the combination of glasdegib and LDAC conferred superior overall survival (OS) versus LDAC alone with a median OS of 8.3 versus 4.3 months.(157). Thus, glasdegib was approved both for USA in 2019 and Europe 2020, but it is currently not available in Spain.

1.4.3. Targeted therapy

Several new drugs have been developed for the treatment of AML targeting different pathways involved in the pathogenesis of the disease, for both first line or the relapse/refractory setting. They are in different stages of investigation, from early phase 1 clinical trials, to already part of the standard of care.

The following figure and table summarize the mechanism of action and results of the (currently) most relevant drugs. For the interest of the present thesis, the drugs targeting FLT3 and NPM1 will be detailed in depth.

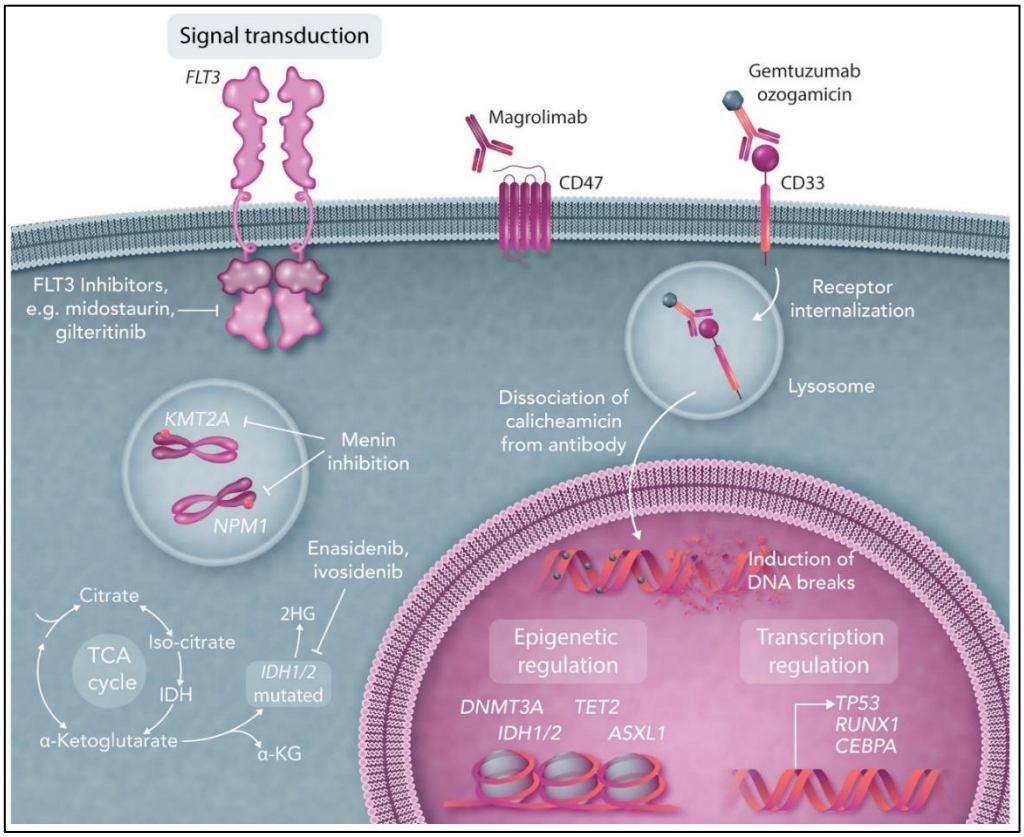


Figure from Kayser S. and Levis M. The clinical impact of the molecular landscape of acute myeloid leukemia (Haematologica 2023)

Table 2: Summary of the most relevant target drugs for AML

Drug	Mechanism of action	Indication	Results
Gemtuzumab ozogamicin (GO)	Anti-CD33 antibody-drug conjugate	Newly diagnosed CD33+ adult AML with intensive CT	Newly diagnosed: CT + GO vs CT alone: median EFS 15.6 vs 9.7 months.(158-161)
Ivosidenib	IDH1 inhibitor	Newly diagnosed *IDH1* mutated AML in patients ≥75 years or ineligible for intensive CT ≥18 years with R/R *IDH1* mutated AML	Newly diagnosed aza-ivo vs aza placebo: CR rate: 47% vs. 15%; median OS 24 vs. 7.9 months.(155) Ivo monotherapy for R/R AML: ORR 41.6%, CR rate 21.6%; median OS 8.8 months.(162)
Enasidenib	IDH2 inhibitor	R/R *IDH2* mutated AML	Aza-ena in ≥18years: ORR 74%; CR rate 19%; median OS 9.3 months(163) Monotherapy >60years vs aza/LDAC/support: ORR (40.5% vs 9.9%) but median OS 6.5 vs 6.2 months (p= 0.23).(164)
Magrolimab	Anti-CD47 antibody	Newly diagnosed *TP53* mutated AML	Phase 1 with aza ORR of 48.6%, median OS of 10.8 months, patients who proceeded to alloHCT 1-year OS of 63%.(165) Phase 3 ongoing.
Eprenetapopt (APR-246)	Small-molecule p53 protein re-conformation	Newly diagnosed *TP53* mutated AML	ORR in AML 64%, CR 36% and median OS 10.8 months.(166) Median OS 13.9 and 3.0 months in AML with ≤ and > 30% marrow blasts.(167) Better results in MDS, Phase 3 pending.

- FLT3 inhibitors

Midostaurin

Midostaurin is a first-generation FLT3 inhibitor, with multi kinase inhibitory effect over different protein kinases such as FLT3, KIT, or PDGFR. The RATIFY phase III trial (168) demonstrated a significant improvement in OS with a median OS of 74.7 months for the midostaurin arm vs 25.6 months in the placebo arm with the addition of midostaurin to standard 7 plus 3 chemotherapy in fit *FLT3*mut AML patients. Based on this finding, in 2017 midostaurin was the first tyrosine kinase inhibitor (TKI) approved to be administered in combination with standard intensive chemotherapy for adult patients with newly diagnosed *FLT3*mut AML. Since 2017, the CETLAM group incorporated midostaurin to the AML-12 protocol for fit adults with AML and *FLT3* mutations.

Gilteritinib

Gilteritinib is a highly selective, potent oral FLT3 inhibitor with activity against ITD and TKD mutations. It is currently the only approved TKI for the treatment of relapsed/refractory *FLT3*mut AML in the USA and Europe based on the results of the ADMIRAL trial: a randomized, open-label, multicenter phase III trial for relapsed/refractory *FLT3*mut patients, who were randomized 2:1 to receive gilteritinib or salvage chemotherapy.(169) In this trial the median OS in the gilteritinib group was significantly longer than that in the chemotherapy group (9.3 months vs. 5.6 months) while the median event-free survival (EFS) was 2.8 months vs 0.7 months respectively. Several trials are currently ongoing with different combinations of gilteritinib for untreated AML (ie: aza-ven-gilteritinib or intensive CT+gilteritinib) or studying its benefit as post-remisison maintenance therapy.

Quizartinib

The QuANTUM-First trial tested the addition of quizartinib (an oral, highly potent, selective FLT3 inhibitor) to standard chemotherapy for newly diagnosed AML patients. Recently published results showed an improved median OS of 31.9 months (95% confidence interval (CI) 21 months-not estimable) for quizartinib versus 15.1 months (95%CI 13.2-26.2) for placebo.(170)

Other FLT3 inhibitors

Sorafenib was initially developed as a targeted drug for the MAPK pathway and it has been studied in several solid neoplasms (ie: hepatocarcinoma, renal cell carcinoma). In Phase 1 studies sorafenib showed significant response in AML *FLT3*mut patients. A few studies in newly diagnosed AML analyzed the benefit of CT+/-sorafenib, and showed promising EFS and relapse rates but similar OS. (171, 172) Currently, sorafenib is used as an off-label monotherapy for post-transplant maintenance.

Crenolanib is being studied both for the relapsed/refractory (R/R) setting in combination with salvage chemotherapy as well as in newly diagnosed *FLT3*mut AML. In the latter setting, in a phase 2 trial a CR of 85% was observed, and with a median follow-up of 45 months the median OS for all patients had not been reached with 57% of patients alive.(173) Phase 3 trials with crenolanib are ongoing.

- Menin inhibitors

Menin is essential for the proliferation and survival of *KMT2A*-rearranged and *NPM1*-mutated AML. *NPM1* mutations have also been associated with the upregulation of *HOXA* genes, similar to gene expression patterns observed in patients with *KMT2A* rearrangements.(174) These findings have led to the hypothesis that AML patients with *NPM1* mutations might also benefit from menin inhibition. Four different menin-MLL1 inhibitors are currently in early-phase clinical trials. Revumenib is one of the most advanced in its development, and results from the Phase 1 have been recently published showing 30% rate of complete remission with complete or partial hematologic recovery and adequate tolerability.(175)

2. HYPOTHESIS

Acute myeloid leukemia (AML) is a highly heterogeneous disease with a complex mutational landscape. The advances in genetic techniques currently provide clinicians with in-depth information about each AML patient that allows for more refined risk stratifications and treatment individualization by identifying patients susceptible to receiving targeted therapy or entering clinical trials. However, although the latest risk-stratification guidelines include several mutations as prognostic markers, the survival impact of gene-gene associations is still difficult to assess.

The first hypothesis of this doctoral thesis is that the presence of *DNMT3A* mutations at AML diagnosis modifies the outcome of patients with *NPM1* and *FLT3* mutations by significantly altering survival and relapse rates.

The second hypothesis of this thesis is that the addition of FLT3 inhibitors to intensive therapy improves the poor outcome associated with AML with *FLT3* mutations in all its molecular subsets.

3. OBJECTIVES

Main objective:

The main objective of the research project presented here is to refine the use of some prognostic markers to accurately predict the outcome of patients with AML in order to apply the best risk-directed therapeutic strategy. Thus, this thesis analyzes the impact of the co-mutational status and the incorporation of FLT3 inhibitors on the outcome of patients with AML with *NPM1* and *FLT3* mutation.

Secondary objectives:

1. To analyze the prognostic impact of *DNMT3A* co-mutation in AML-*NPM1* patients with and without *FLT3* mutations.
2. To study how the presence of *DNMT3A* mutation affects the evolution of the molecular measurable residual disease of *NPM1* mutated AML.
3. To assess the change in the prognosis of patients with AML and *FLT3* mutations before and after the incorporation of the FLT3 inhibitor midostaurin in a homogeneous prospective protocol.
4. To analyze the impact of midostaurin among the different molecular subgroups of *FLT3* mutated AML.

4. COMPENDIUM OF PUBLICATIONS

4.1. Article 1

Prognostic impact of *DNMT3A* mutation in acute myeloid leukemia with mutated *NPM1*

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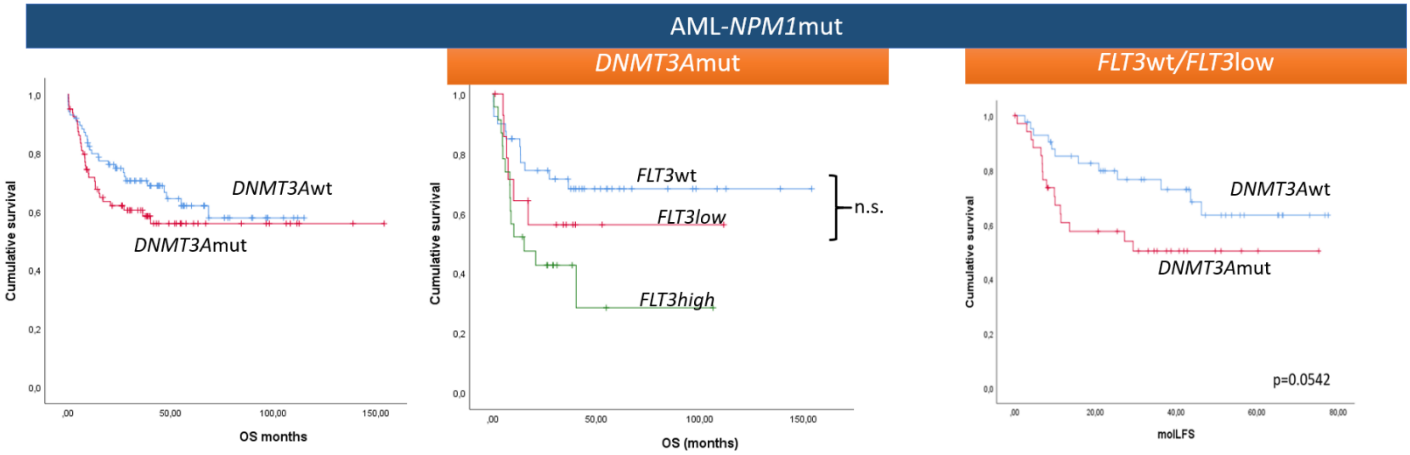
Visual abstract:



REGULAR ARTICLE

blood advances

Prognostic impact of *DNMT3A* mutation in acute myeloid leukemia with mutated *NPM1*

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Key Points

- Patients with *DNMT3A*^{mut} have worse *NPM1* MRD clearance, which can be counteracted by preemptive allogeneic transplantation.
- *DNMT3A*^{mut} does not modify the prognostic value of the *FLT3*-ITD allelic ratio in AML-*NPM1*.

The negative prognostic impact of internal tandem duplication of *FLT3* (*FLT3*-ITD) in patients with acute myeloid leukemia with mutated *NPM1* (AML-*NPM1*) is restricted to those with a higher *FLT3*-ITD allelic ratio (*FLT3*^{high}; ≥0.5) and considered negligible in those with a wild-type (*FLT3*^{WT})/low ITD ratio (*FLT3*^{low}). Because the comutation of *DNMT3A* (*DNMT3A*^{mut}) has been suggested to negatively influence prognosis in AML-*NPM1*, we analyzed the impact of *DNMT3A*^{mut} in *FLT3*-ITD subsets (absent, low, and high ratios). A total of 164 patients diagnosed with AML-*NPM1* included in 2 consecutive CETLAM protocols and with *DNMT3A* and *FLT3* status available were studied. Overall, *DNMT3A*^{mut} status did not have a prognostic impact, with comparable overall survival ($P = .2$). Prognostic stratification established by *FLT3*-ITD (*FLT3*^{WT} = *FLT3*^{low} > *FLT3*^{high}) was independent of *DNMT3A*^{mut} status. Measurable residual disease (MRD) based on *NPM1* quantitative polymerase chain reaction was available for 94 patients. *DNMT3A*^{mut} was associated with a higher number of mutated *NPM1* transcripts after induction ($P = .012$) and first consolidation (C1; $P < .001$). All *DNMT3A*^{mut} patients were MRD$^+$ after C1 ($P < .001$) and exhibited significant MRD persistence after C2 and C3 (MRD$^+$ vs MRD$^-$; $P = .027$ and $P = .001$, respectively). Finally, *DNMT3A*^{mut} patients exhibited a trend toward greater risk of molecular relapse ($P = .054$). In conclusion, *DNMT3A*^{mut} did not modify the overall prognosis exerted by *FLT3*-ITD in AML-*NPM1* despite delayed MRD clearance, possibly because of MRD-driven preemptive intervention.

Introduction

In recent years, the role of molecular genetics has proven to be essential in deciphering the heterogeneity of acute myeloid leukemia (AML)[1,2] and defining genetic markers of prognostic significance that can guide risk-adapted treatment.[3]

AML with mutations in the nucleophosmin 1 gene (AML-*NPM1*) forms a specific category in the latest World Health Organization classification because of its singular characteristics.[4] The cooccurrence of

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mutated *NPM1* (*NPM1*^{mut}) and the internal tandem duplication of *FLT3* (*FLT3*-ITD) in de novo AML with intermediate-risk cytogenetics results in a different prognostic impact depending on the *FLT3* allelic burden.[5-7] Previous studies have shown that patients with *NPM1*^{mut} and an *FLT3*-ITD low ratio (*FLT3*^{low}; *FLT3*-ITD/FLT3^{WT} ratio of <0.5) had overall survival (OS) and risk of relapse (RR) similar to those of patients with *NPM1*^{mut} and wild-type (WT) *FLT3* (*FLT3*^{WT}).[8,9] Since 2012, these findings have been included in our latest protocol (CETLAM [Spanish Cooperative Group for the Diagnosis and Treatment of Acute Myeloid Leukemia and Myelodysplastic Syndromes] AML-12), and patients with *FLT3*^{low}-*NPM1*^{mut} AML are not considered for allogenic hematopoietic stem cell transplantation (alloHSCT) in first complete remission (CR1). However, a molecular-based measurable residual disease (MRD) monitoring protocol is strictly followed to allow early-intervention strategies.

The DNA (cytosine-5)-methyltransferase 3A (*DNMT3A*) gene is located on the short arm of chromosome 2 and encodes for a DNA methyltransferase that methylates unmodified DNA cytosine residues modulating the expression of several genes.[10,11] Almost all *DNMT3A* mutations are heterozygous, and more than two-thirds cluster at the methyltransferase domain in codon R882, causing loss of methylation activity by disturbing *DNMT3A* tetramerization.[12-16] However, although a precise methylation pattern alteration resulting from mutations in *DNMT3A* has not yet been established,[17-21] a new mechanism of leukemogenesis characterized by the upregulation of the hepatic leukemia factor (a specific leukemic transcription factor) has been shown to be related to the cooccurrence of *DNMT3A*, *NPM1*, and *FLT3* mutations.[22]

DNMT3A is considered a founder mutation in AML.[23,24] It has been associated with age-related clonal hematopoiesis, with increasing frequency in healthy elderly individuals,[25] although a recent study found a correlation of *DNMT3A* mutations with younger age in *NPM1*^{mut} AML.[26] Patients with AML and mutated *DNMT3A* (*DNMT3A*^{mut}) are frequently older and present with higher white blood cell (WBC) counts and higher platelet counts compared with WT *DNMT3A* (*DNMT3A*^{WT}).[13,17,27] *DNMT3A* is the third most frequently mutated gene in AML patients included in intensive chemotherapy trials. It is predominantly observed in AML-*NPM1* (73%) and less frequently in patients with mutations in chromatin remodeling genes or genes involved in spliceosome function. Interestingly, a recurrent association of *NPM1*^{mut}/*DNMT3A*^{mut}/*FLT3*-ITD has been observed in 6% of AML cases.[28,29] The prognostic significance of mutations in *DNMT3A* has been controversial; some studies have found no significant influence on survival outcomes,[17] whereas others have suggested that the cooccurrence of *NPM1*^{mut}/*DNMT3A*^{mut}/*FLT3*-ITD in AML patients is associated with adverse outcomes.[7,13,30-33]

In 2013, the German AML Study Group described the clinical impact of *DNMT3A*^{mut} in younger adults with AML.[34] In a univariable exploratory subset analysis, the group showed a significant prognostic impact of *DNMT3A*^{mut} in unfavorable European LeukemiaNet (ELN) AML, whereas no impact was observed in favorable ELN AML. In 2016, the proposed AML gene classification by Papaemmanuil et al[28] showed a deleterious effect of *DNMT3A*^{mut} when specifically associated with *FLT3*-ITD independently of its allelic ratio.

We analyzed whether this triple-gene alteration led to an unfavorable prognosis in AML-*NPM1* patients, with particular attention to those harboring *FLT3*^{low}.

Methods

Patients and samples

Patients with de novo AML and intermediate-risk cytogenetics according to the Medical Research Council,[35] *NPM1*^{mut}, and available bone marrow sample from diagnosis were selected. All patients were diagnosed between 2003 and 2017 and were included in the CETLAM intensive treatment protocols AML-03 and AML-12 (registered at www.clinicaltrials.gov as #NCT01723657 and #NCT04687098) provided they met the criteria for eligibility. The present study was reviewed and approved by the ethics committee of the Hospital de la Santa Creu i Sant Pau (Comitè ètic d'Investigació Clínica). Informed consent for both bone marrow analysis and treatment was obtained in all cases according to the Declaration of Helsinki.

Molecular studies

Diagnostic bone marrow samples from all patients were analyzed for *DNMT3A*^{mut} as previously described.[17] The mutational status of the *FLT3* gene was also established. In mutated cases, the allelic ratio was calculated by dividing the area under the curve of the *FLT3*-ITD peak and the area under the curve of the *FLT3*^{WT} peak. Patients were stratified into 2 groups: those with a high ratio (*FLT3*^{high}) if the ratio of *FLT3*-ITD/*FLT3*^{WT} was ≥0.5 and those with *FLT3*^{low} if the ratio of *FLT3*-ITD/*FLT3*^{WT} was <0.5.

Monitoring of *NPM1* MRD was performed on bone marrow samples by quantitative reverse transcription polymerase chain reaction (sensitivity 10^{-4} to 10^{-6}) as previously described.[36] After each treatment cycle, absolute transcript reduction was estimated, and its logarithm (log10) reduction from diagnosis was also explored. Based on the latest ELN MRD working party recommendations,[37] MRD positivity was considered when *NPM1* transcripts were amplified in at least 2 of 3 replicates with cycle threshol values of ≤40 at a cycling threshold of 0.1. Molecular relapse was confirmed if the MRD level (in a patient previously MRD$^-$) increased ≥1 log10 between 2 consecutive positive samples, and molecular progression was confirmed if copy numbers increased ≥1 log10 between 2 positive samples in a patient with MRD$^+$.

Statistical analysis

Analysis of the relationship between categorical variables was performed using the χ^2 or Fisher's exact test. Differences between groups for continuous variables were established through the independent samples *t* test or Mann-Whitney U test. All tests were 2 sided and considered significant where $P < .05$. OS was calculated from diagnosis to death, whereas leukemia-free survival (LFS) was calculated from CR to death or relapse; both functions were estimated with the Kaplan-Meier method. Unless specified otherwise, all survival results reported reflect 5-year estimates. A log-rank test was run to determine differences in the survival distribution, with a significance threshold of $P \leq .05$. RR was estimated using the cumulative incidence method, defining relapse as the main event and death without relapse as the competitive event. Molecular LFS (molLFS) was estimated from CR to molecular failure, overt hematological relapse, or death. All statistical analyses were performed with SPSS software (version 26; IBM, Armonk, NY) and R statistics (version 3.6.1; R Foundation for Statistical Computing, Vienna, Austria).

Table 1. Patient characteristics according to *DNMT3A*^{mut} status

	No. (%)		
	DNMT3A^{WT} (n = 85)	*DNMT3A*^{mut} (n = 79)	*P*
Female sex	51 (60)	36 (46)	.08
Age, y			.7
Median	53	53	
Range	18-71	25-72	
WBC, × 10^9/L			<.001
Median	16	50	
Range	0.55-408	1.3-384	
BM blasts, %			.7
Median	73	73	
Range	20-100	20-100	
Platelet count, × 10^9/L			.4
Median	64	70	
Range	8-488	12-625	
***FLT3* mutational status**			.6
WT	46 (54)	40 (50)	
FLT3^{low}	19 (22)	15 (19)	
FLT3^{high}	19 (22)	23 (30)	
Treatment protocol			
AML-03	26 (31)	22 (28)	
AML-12	59 (69)	57 (72)	
Postinduction CR	75 (88)	69 (87)	.7
No. of cycles to achieve CR (1 vs 2)	67 vs 8	61 vs 8	.86
No. of patients undergoing alloHSCT in CR1	16 (19)	22 (28)	.9

BM, bone marrow.

Results

Patient characteristics according to *DNMT3A*^{mut} status

A total of 164 patients with AML-*NPM1* were included. Clinical and genetic characteristics at diagnosis are described in Table 1. Patients were included in protocols AML-03 (n = 48) and AML-12 (n = 116). *DNMT3A*^{mut} was found in 79 patients (48%); in 62 cases (38%), mutations were in codon R882 or were insertions/deletions, whereas 17 (10%) were different missense mutations. Seventy-six patients (46%) harbored *FLT3*-ITD, 42 of whom had *FLT3*^{high} (55%). According to *DNMT3A*^{mut} status, patient characteristics were comparable, except for a higher WBC presentation among *DNMT3A*^{mut} patients. Of note, the proportion of *FLT3*-ITD allelic burden subsets (ie, *FLT3*^{WT}, *FLT3*^{low}, and *FLT3*^{high}) was independent of *DNMT3A*^{mut} (*P* = .6).

Eighty-eight percent of patients achieved CR after 1 or 2 cycles of induction therapy (n = 128 and 16, respectively); 6% (n = 10) had refractory disease, and 10 patients died during induction. As consolidation therapy, 58 patients received intensive treatment consisting of 2 to 3 high-dose cytarabine cycles. AlloHSCT was performed in 65 patients (CR1, n = 44; CR2, n = 15; with refractory disease, n = 4); 14 patients underwent autologous transplantation. The median follow-up time was 30 months.

Prognostic impact of *DNMT3A*^{mut}

In the entire cohort of AML-*NPM1* patients, OS, LFS, and RR were 59% ± 4%, 60% ± 5%, and 27% ± 7%, respectively. *FLT3*-ITD allelic ratio confirmed its prognostic impact, with a similar outcome for patients with *FLT3*^{WT} or *FLT3*^{low} and a significantly worst prognosis for cases with *FLT3*^{high}. OS was 67% ± 6% vs 62% ± 9% vs 40% ± 8% (*P* = .002; supplemental Figure 1), respectively; RR was 18% ± 9% vs 27% ± 16% vs 41% ± 17% (*P* = .008), respectively; and LFS was 71% ± 5% vs 56% ± 9% vs 40% ± 9% (*P* = .002), respectively. In contrast, *DNMT3A*^{mut} did not exert a significant effect on overall outcomes (Figure 1), with OS in *DNMT3A*^{WT} vs *DNMT3A*^{mut} patients of 62% ± 6% vs 56% ± 6% (*P* = .2), respectively; LFS of 65% ± 6% vs 54% ± 6% (*P* = .1), respectively; and RR of 22% ± 11% vs 31% ± 11% (*P* = .2). The outcomes of *DNMT3A* subsets among the entire AML-12 cohort are available in supplemental Figure 2.

Additionally, the individual effect of R882 *DNMT3A*^{mut} was analyzed separately and did not show any prognostic impact (*P* = .4; supplemental Figure 3). Multivariate analysis performed for OS included age, protocol, WBC count, *DNMT3A*^{mut} status, and *FLT3*-ITD subsets, the latter being the only statistically significant variable (data not shown).

The effect of the cooccurrence of *DNMT3A*^{mut} and *FLT3*-ITD was analyzed separately. In the *DNMT3A*^{mut} cohort, whereas OS between *FLT3*^{WT} and *FLT3*^{low} was similar, patients with *FLT3*^{high} showed particularly worse outcomes (*FLT3*^{WT} vs *FLT3*^{low} OS, 68% ± 8% vs 56% ± 13%, respectively; *P* = .3; *FLT3*$^{WT/low}$ vs *FLT3*^{high} OS, 65% ± 7% vs 28% ± 13%, respectively; *P* = .015). This was further validated in terms of LFS (*FLT3*^{WT} vs *FLT3*^{low}, 68% ± 8% vs 50% ± 13%, respectively; *P* = .1; *FLT3*$^{WT/low}$ vs *FLT3*^{high}, 62% ± 7 vs 20% ± 16%, respectively; *P* = .014) and RR in the same groups (23% ± 15% vs 29% ± 23%, respectively; *P* = .4; 25% ± 13% vs 48% ± 20%, respectively; *P* = .017; Figure 2).

In patients without *DNMT3A*^{mut}, the allelic ratio of *FLT3*-ITD maintained its prognostic value, with similar outcomes between *FLT3*^{WT} and *FLT3*^{low} patients. Interestingly, in this group of patients, the deleterious effect of *FLT3*^{high} was mildly modulated (*FLT3*^{WT} vs *FLT3*^{low} OS, 66% ± 8% vs 66% ± 11%, respectively; *P* = .5; *FLT3*$^{WT/low}$ vs *FLT3*^{high} OS, 66% ± 7% vs 47% ± 12%, respectively; *P* = .028). Similar findings were seen in terms of LFS (*FLT3*^{WT} vs *FLT3*^{low}, 74% ± 8% vs 61% ± 13%, respectively; *P* = .2; *FLT3*$^{WT/low}$ vs *FLT3*^{high}, 70% ± 7% vs 52% ± 13%, respectively; *P* = .083) and RR (14% ± 11% vs 26% ± 21%, respectively; *P* = .2; 17% ± 12% vs 33% ± 23%, respectively; *P* = .1; Figure 3).

The direct comparison of *DNMT3A*^{mut} status according to each *FLT3* status (WT, low, and high) was not statistically different (supplemental Figure 4), although the general analysis of *DNMT3A* depending on *FLT3*^{WT} or *FLT3*^{mut} showed statistical differences. This might be due to the low number of patients analyzed when dividing into the 3 groups.

MRD kinetics according to *DNMT3A*^{mut} status

Patients included in the CETLAM AML-12 trial with AML-*NPM1* allocated to the favorable ELN category (*FLT3*^{WT} or *FLT3*^{low}) were not intended to undergo alloHSCT in CR1 unless a molecular failure was identified. Therefore, patients were closely monitored for *NPM1* MRD at specific time points (postinduction, after each consolidation

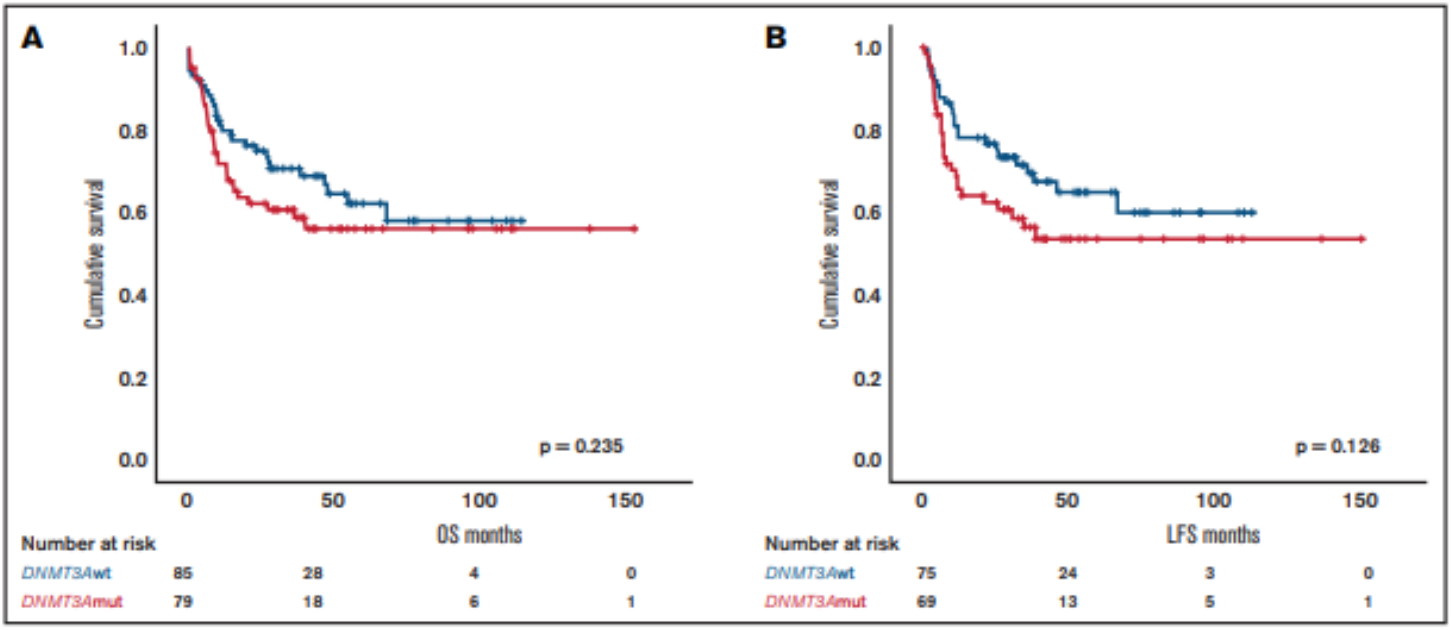

Figure 1. Overall impact of *DNMT3A*^{mut} on AML-*NPM1*. OS (A) and LFS (B) of patients according to *DNMT3A*^{mut} status.

cycle, and every 3 months for 3 years after treatment completion) to ensure rapid detection of molecular relapse and subsequent therapy initation.[38] Patients with *FLT3*^{high} underwent alloHSCT after the first consolidation cycle (C1).

In 94 patients with available MRD data, we further investigated whether *DNMT3A*^{mut} status influenced *NPM1* MRD. Although the probability of achieving hematological CR was not affected by *DNMT3A* (75 vs 69 patients in with *DNMT3A*^{WT} and *DNMT3A*^{mut}, respectively; $P = .46$), *NPM1* MRD kinetics differed according to *DNMT3A*^{mut} status. Patients with *DNMT3A*^{mut} showed a higher number of absolute *NPM1*^{mut} transcripts after induction ($P = .012$) and C1 ($P < .001$; Figure 4A). Similar findings were observed after C2 and C3 among patients not intended to undergo alloHSCT in CR1.

Therefore, we explored the relationship between *DNMT3A* subsets, posttreatment molecular MRD status (positive vs negative), and MRD response depth (log10 reduction; Figure 4B-G). After induction, all but 1 patient remained MRD$^+$. Although there were no statistical differences in the number of log10 reductions, a trend toward a more profound molecular response (≥4 log10) was observed in the *DNMT3A*^{WT} group (*DNMT3A*^{WT} vs *DNMT3A*^{mut}, 39% vs 15%, respectively; *P* not significant).

After C1, none of the *DNMT3A*^{mut} patients achieved MRD$^-$ status, compared with 32% of *DNMT3A*^{WT} patients ($P = .001$). Of note, patients without *DNMT3A*^{mut} presented a deeper MRD reduction (≥4 log10 reduction in 77% of *DNMT3A*^{WT} vs 46% of *DNMT3A*^{mut} patients; $P = .033$). The relationship between *DNMT3A* and *NPM1* MRD was also sustained after C2 and C3 (Figure 4B-G; supplemental Figure 5). Additionally, when considering the triple-mutated group (*NPM1*, *FLT3*-ITD, and *DNMT3A*), all patients remained MRD$^+$ after induction, C1, and C2 regardless of *FLT3*-ITD allelic ratio.

Finally, the potential influence of *DNMT3A*^{mut} status on molecular failure was explored. Among 85 cases included in the AML-12 protocol not initially considered for alloHSCT in CR1 (AML-*NPM1* with *FLT3*^{WT} or *FLT3*^{low}, n = 63 and 22, respectively), the median molLFS was not reached at a mean follow-up of 30 months (supplemental Figure 6). When stratified by *DNMT3A*^{mut} status, patients with the WT form exhibited a trend toward a long-term sustained molecular CR (molLFS, 63% ± 9% vs 50% ± 9% in *DNMT3A*^{WT} (n = 42) vs *DNMT3A*^{mut} (n = 35), respectively; $P = .054$; Figure 5).

Eleven patients in the favorable-risk group harbored *NPM1*^{mut}/*FLT3*^{low}/*DNMT3A*^{mut}; of these, only 5 experienced a molecular or hematological relapse and underwent alloHSCT. In total, 23 patients (27%) in this favorable subgroup underwent alloHSCT because of molecular or hematological relapse.

Overall, these findings suggest a deleterious effect of *DNMT3A*^{mut} on *NPM1* MRD that should be validated in larger studies.

Discussion

Several studies have been published attempting to elucidate the prognostic impact of *DNMT3A*^{mut}, but many have had contradictory results. This may be due to differences in the biological characteristics of the patients included (age, cytogenetics, availability of molecular studies), the treatment protocols, or other factors.[39] Of note, even those studies comparing the impact of *DNMT3A*^{mut} status based on *NPM1*^{mut} and *FLT3*^{mut} status have shown contradictory results.[13,17,34] The aim of this study was not to analyze the impact of *DNMT3A*^{mut} on AML outcomes, but rather to analyze its effect in the particular subset of patients with *NPM1*^{mut} and *FLT3*-ITD, after the publication of a large study showing that *DNMT3A*^{mut} have a deleterious effect on outcomes when cooccurring in this subgroup.[28] Our group described the effect of the *FLT3*-ITD ratio in 2012, and it was incorporated into the new treatment protocol. Consequently, patients with *NPM1*^{mut} and *FLT3*^{low} did not undergo alloHSCT in CR1. Therefore, we had a long follow-up in this group of patients treated following the ELN 2017 recommendations in which to analyze the possible effect of *DNMT3A*^{mut}.

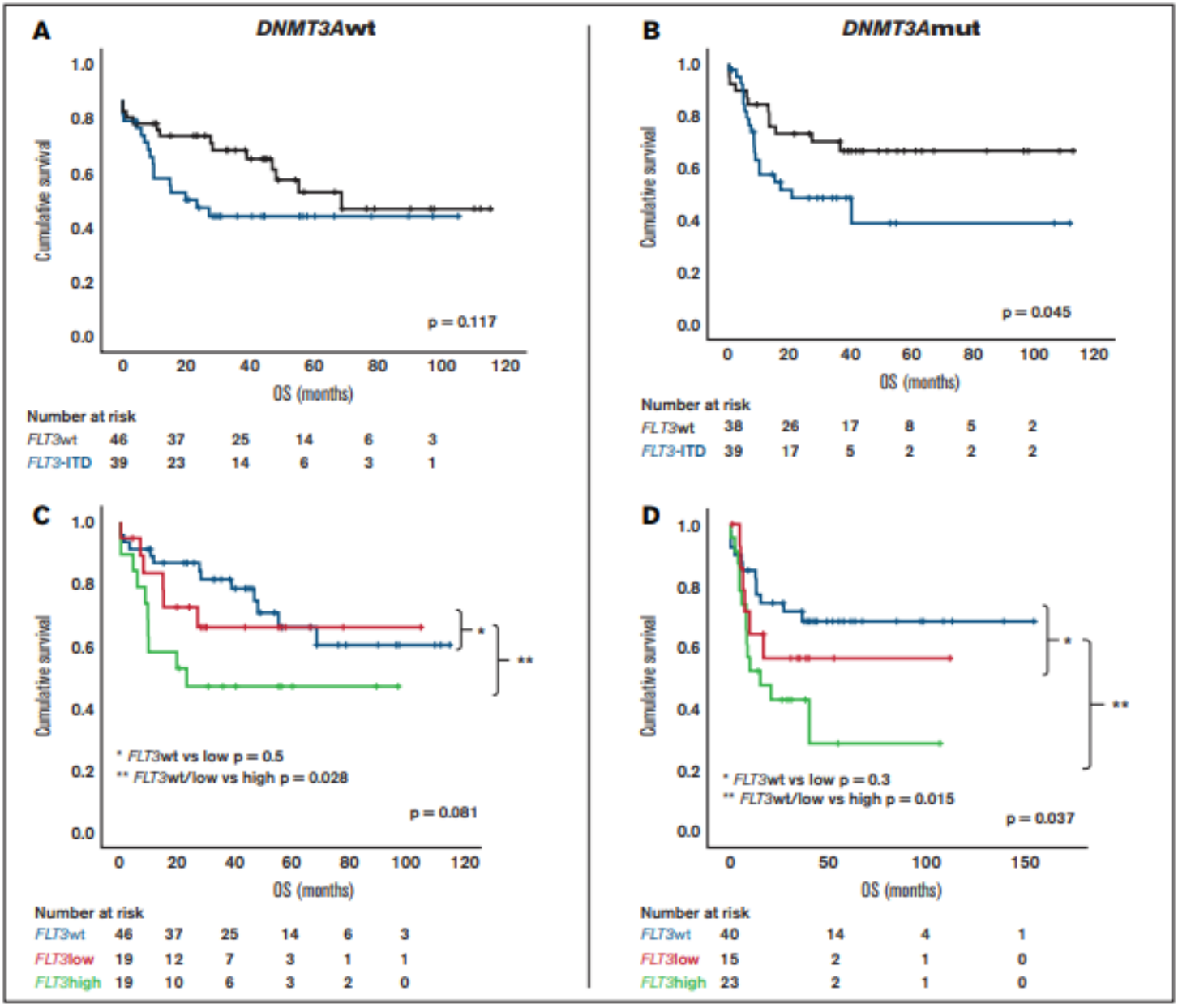

Figure 2. *DNMT3A* influence over *FLT3*-ITD allelic ratio subgroups. OS of *DNMT3A*^{WT} (A,C) and *DNMT3A*^{mut} (B,D) patients in different *FLT3* subsets.

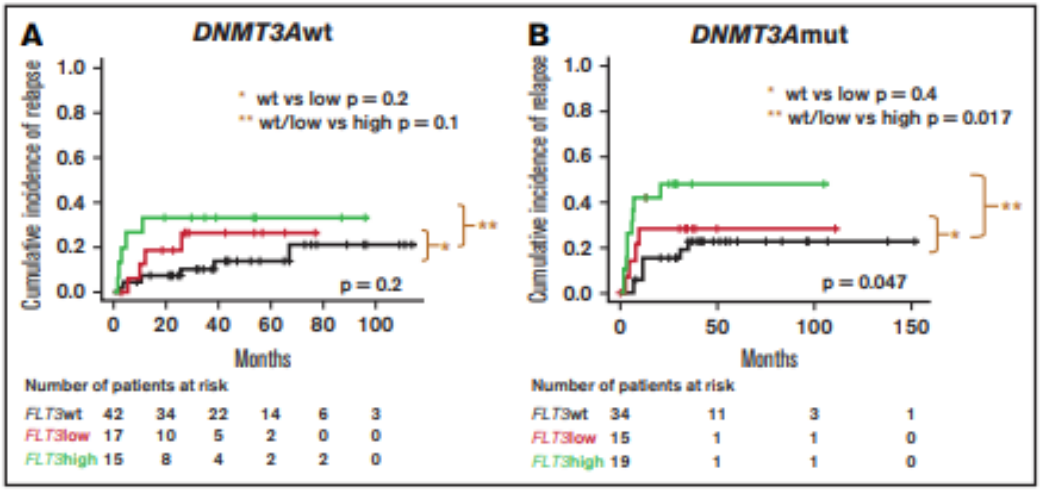

Figure 3. Cumulative incidence of relapse in AML-*NPM1* patients based on *DNMT3A*^{mut} status. *DNMT3A*^{WT} (A) and *DNMT3A*^{mut} (B) patients according to *FLT3*-ITD subgroup.

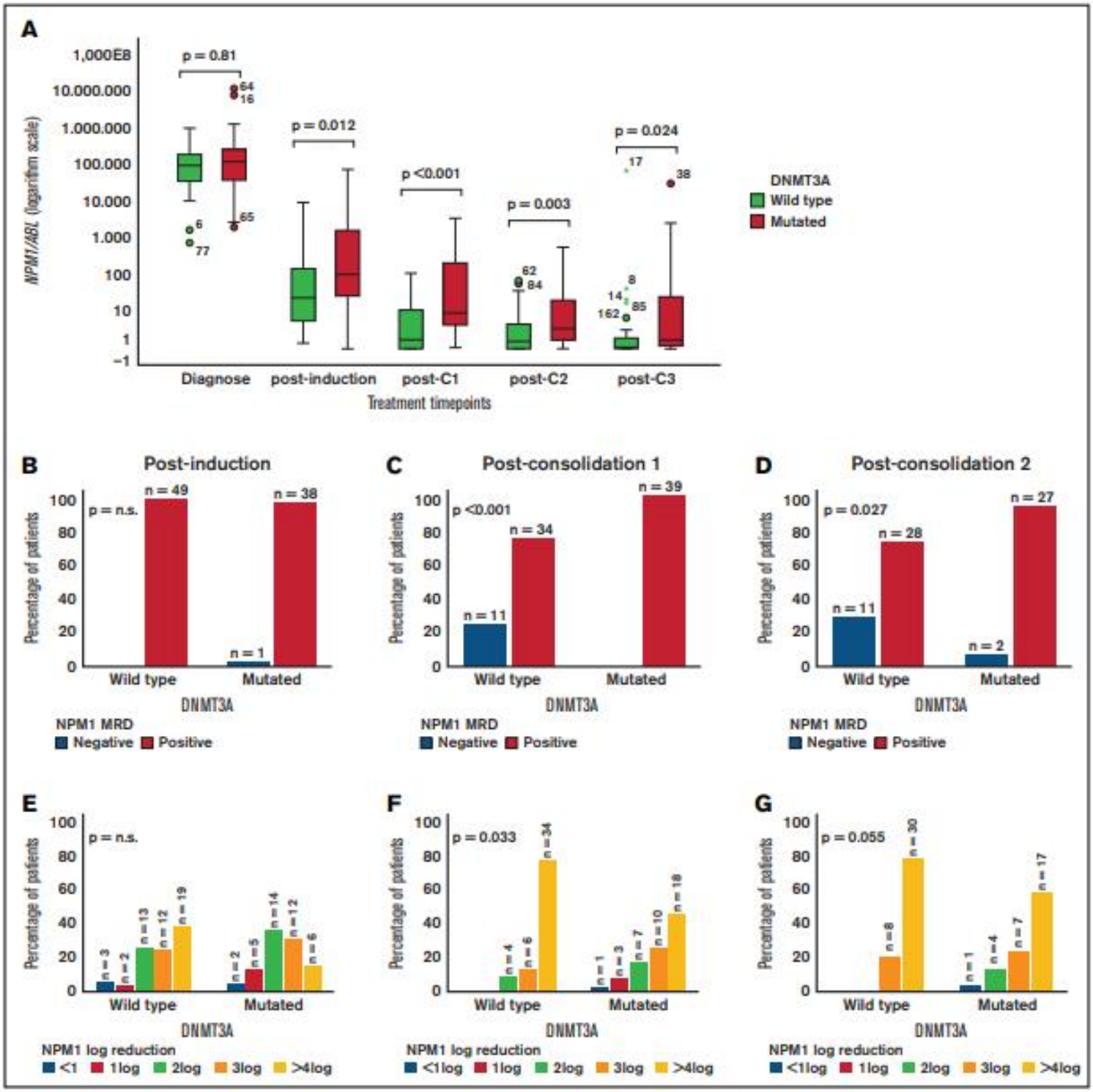

Figure 4. *NPM1* MRD distribution at relevant clinical time points according to *DNMT3A*^{mut} status. (A) *NPM1* absolute transcript distribution in logarithmic scale. (B-G) MRD response, with MRD$^+$ and MRD$^-$ rates (B-D) and corresponding equivalent log10 reductions (E-G) at postinduction (B,E), post-C1 (C,F), and post-C2 (D,G).

In our study, survival analysis showed that *DNMT3A*^{mut} did not have an impact in this particular group and that patients with *NPM1*^{mut} and *FLT3*^{low} had similar outcomes to patients with *NPM1*^{mut} and *FLT3*^{WT} regardless of *DNMT3A*^{mut} status. However, because an effect of *DNMT3A*^{mut} on *NPM1* MRD clearance was demonstrated, we investigated the influence of an early intervention planned in the treatment protocol when molecular relapse was detected. In the last few years, several publications analyzing the prognostic value of MRD follow-up based on *NPM1* transcript levels have been published. Although there is no consensus regarding the cutoff level or evaluation time points, all of them support the prognostic impact of MRD$^+$ persistence, with a higher incidence of relapse and shorter OS.[38,40-43] The largest study performed[44] evaluated the impact of MRD$^+$ in peripheral blood after the second chemotherapy cycle; it found the same impact on prognosis as previously reported, but the authors also reported that MRD persistence was the only independent prognostic factor for death in multivariate analysis. The ELN recommendations[37] also state that in AML-*NPM1*, rising MRD levels or the failure to achieve MRD$^-$ CR is associated with disease relapse and consequently advise that a change in therapy should be

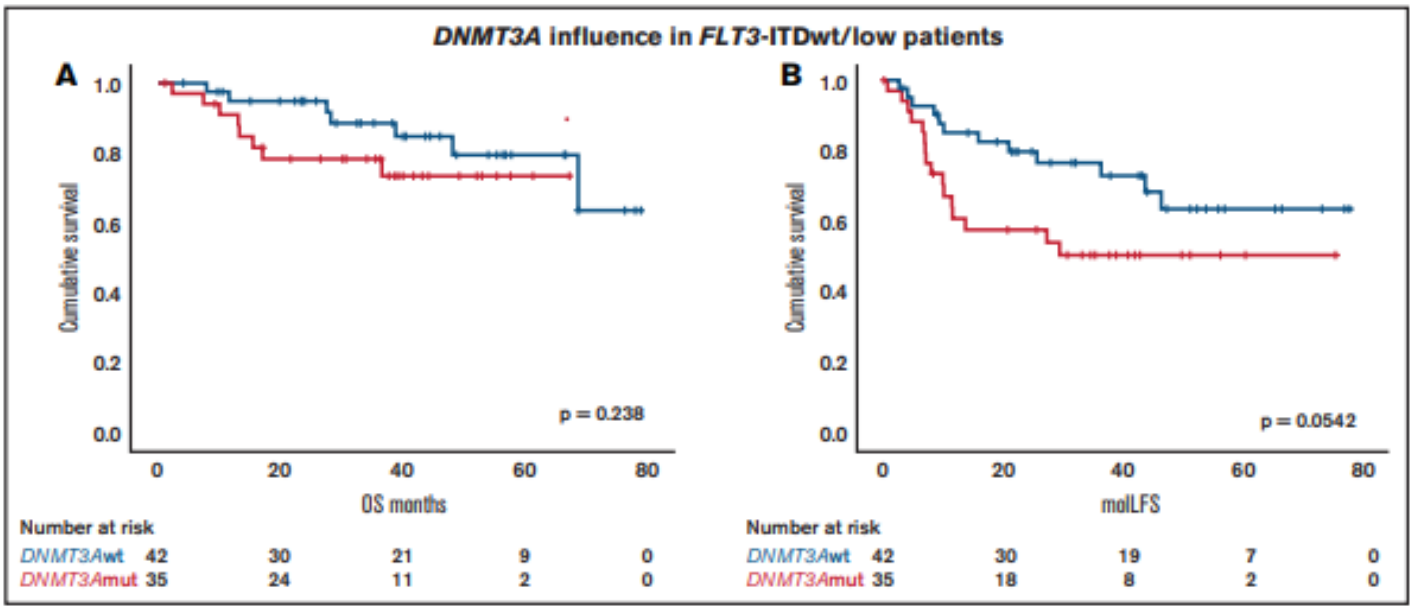

Figure 5. *DNMT3A* influence in the favorable *FLT3*-ITD subgroup. Distribution of patients with $FLT3^{WT}$ or $FLT3^{low}$ according to OS (A) and molLFS (B).

considered. Following the same reasoning, it was recently published by our group that an MRD ratio ($NPM1^{mut}$/ABL1X100) of ≥0.05 (in bone marrow) after the C1 was associated with significantly lower molLFS and that an early intervention resulted in a favorable outcomes.[38] Consequently, using the MRD level to guide postremission therapy can be considered a good strategy.

Interestingly, in the present study, a trend toward worse molLFS was observed in patients with $DNMT3A^{mut}$, but without an impact on OS. When only patients in the favorable ELN 2017 risk group were considered, we found that 27% of patients met either cytological or molecular relapse criteria. Of those, 70% underwent alloHSCT in CR1 (in molecular relapse) or CR2. As a result, the effect of this strategy might counteract the negative effect on OS seen in the $DNMT3A^{mut}$ subgroup. This intervention might be the most important difference between the treatment protocols for our patients and those included in the Papaemmanuil et al[28] study, which considered alloHSCT only in patients at high cytogenetic risk, whereas intermediate-risk patients underwent alloHSCT only when a sibling donor was available.[45-47]

Considering these findings, close MRD monitoring in $DNMT3A^{mut}$ AML-*NPM1* patients, along with early intervention strategies when a molecular relapse is detected, could be an appropriate approach, with a possible impact on OS.

Patients with $DNMT3A^{mut}$ and $FLT3^{high}$ had poorer outcomes than patients in the favorable ELN group (ie, $FLT3^{WT}$ or $FLT3^{low}$). Nonetheless, $DNMT3A^{mut}$ status did not seem to affect patients with $FLT3^{high}$, although a deleterious effect of this triple-mutation status ($NPM1^{mut}$/$FLT3^{high}$/$DNMT3A^{mut}$ vs $NPM1^{mut}$/$FLT3^{high}$/$DNMT3A^{WT}$) cannot be definitively excluded because of the small size of the subgroups analyzed. These findings may show a dosage effect on the interaction between *FLT3* and $DNMT3A^{mut}$ in AML-*NPM1*, highlighting the relevance of considering not only the presence of every single mutation but also the interaction among them.

In conclusion, patients with *NPM1*-AML with $FLT3^{low}$ and $DNMT3A^{mut}$ can be classified as favorable risk, but closer MRD follow-up is recommended to detect a molecular relapse and proceed to a therapeutic intervention.

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G.O. is a PhD candidate at the Autonomous University of Barcelona, and this work is submitted in partial fulfillment of the requirement for a PhD.

Authorship

Contribution: G.O. and A.A. performed research. M.P. and G.O. designed research, analyzed data, and wrote the paper; J.S., J.F.N., and J.E. supervised research and wrote the paper; M.A., S.V., R.C., M.T., A.S., L.E., O.S., A.G., and J.B. collected and provided the clinical data; A.B., A.G., and M.H. analyzed data; and all authors reviewed the final manuscript.

Conflict-of-interest disclosure: J.E. reports an advisory role and trial investigation for Novartis, Daiichi Sankyo, Astellas, Celgene, Jazz Pharmaceuticals, Roche, Boehringer Ingelheim, and Janssen. J.S. reports personal fees from AbbVie, Vyxeos, Gilead, CSL Behring, Astellas, and Gilead; grants and personal fees from Novartis and Daiichi-Sankyo; and grants from Amgen. The remaining authors declare no competing financial interests.

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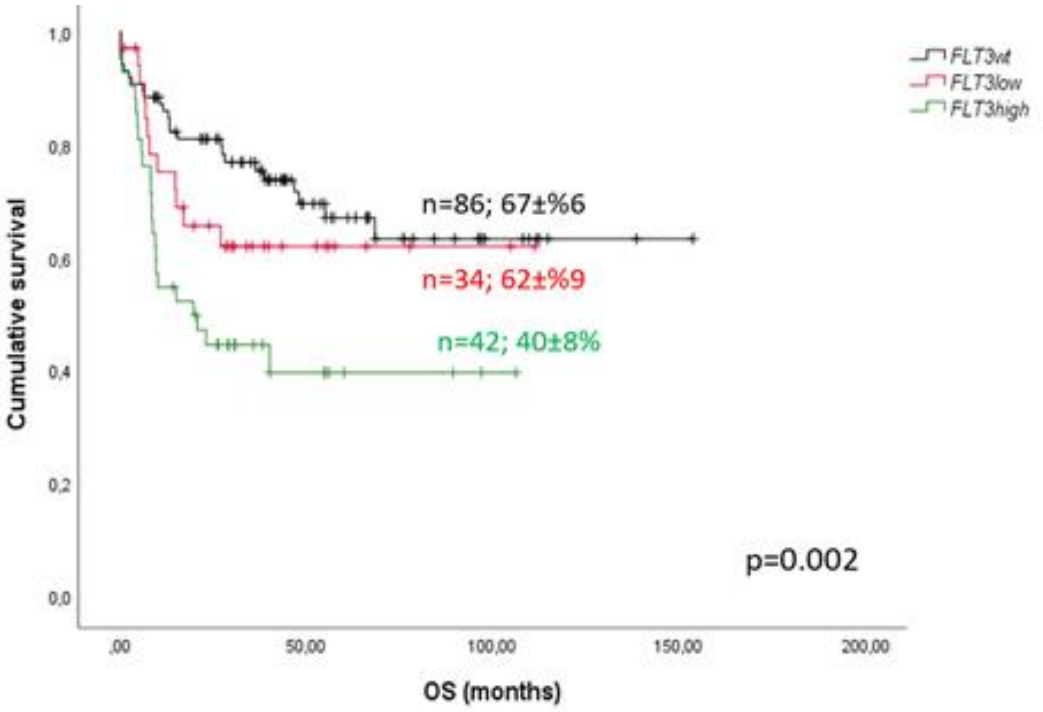
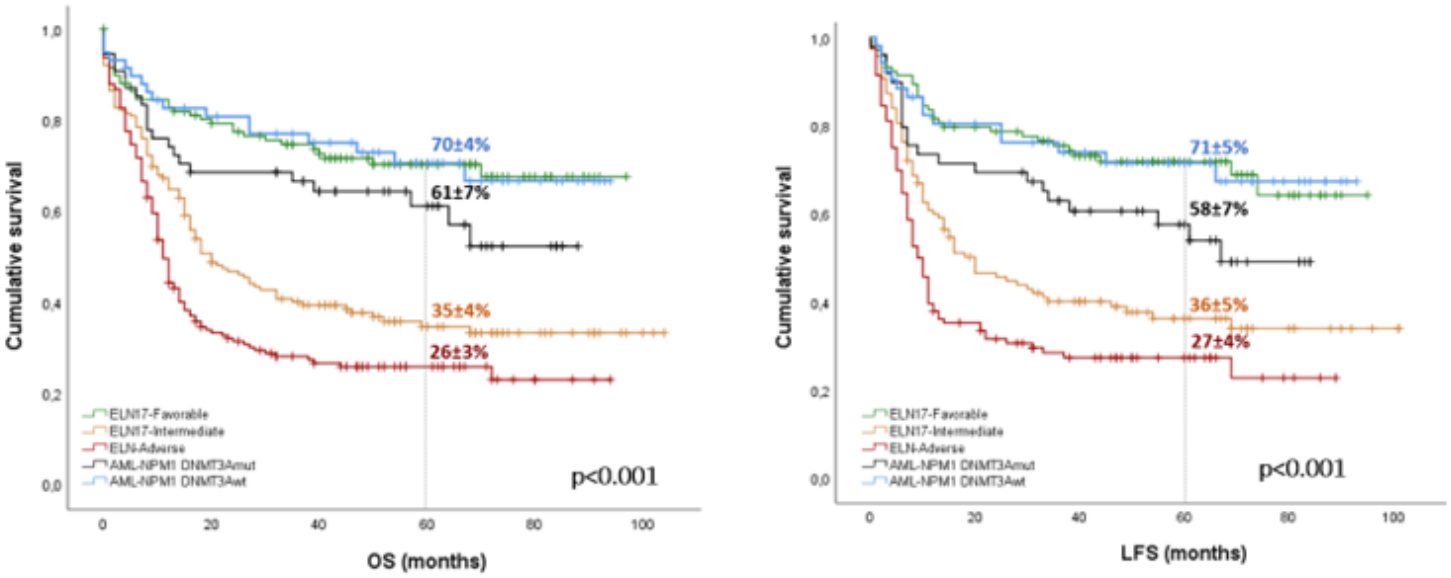
Supplemental Material:

Oñate et al. Prognostic impact of *DNMT3A* in acute myeloid leukemia with mutated *NPM1*.

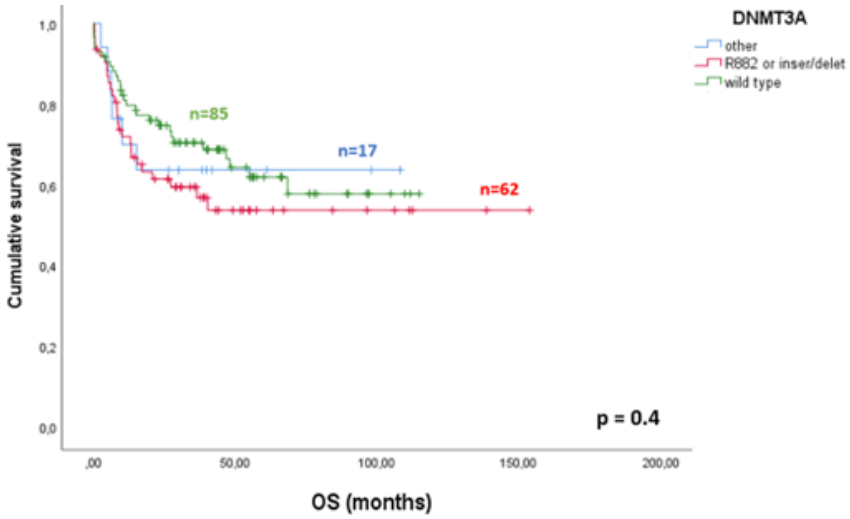
Supplemental Figure 1: Survival impact of *FLT3*-ITD allelic ratio. Overall survival (OS) of AML-*NPM1* patients according to *FLT3*-ITD allelic ratio subgroups



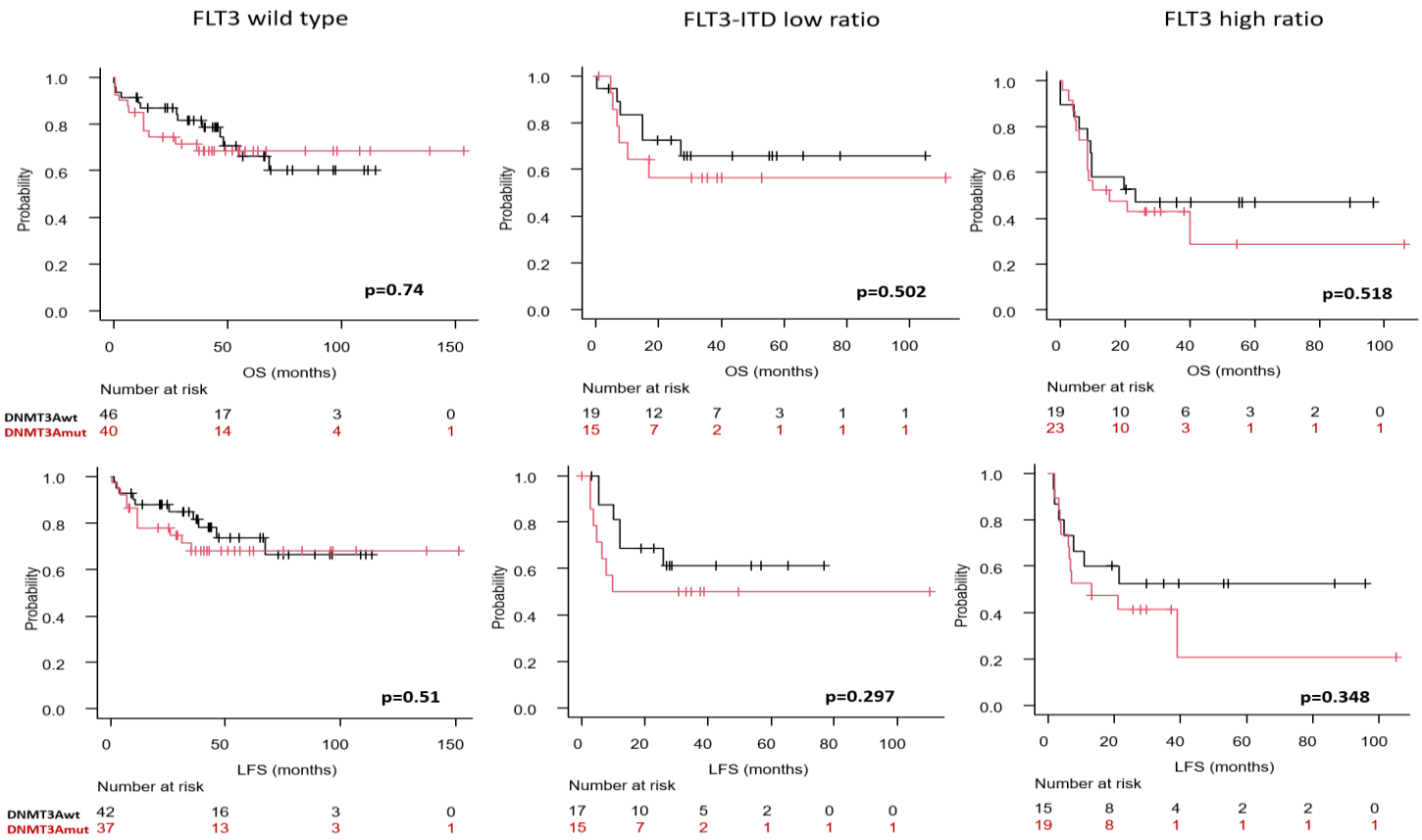
Supplemental figure 2: Survival impact of *DNMT3A* mutational status in AML-*NPM1* contrasted to CETLAM-12 patients. Overall survival and leukemia free survival of AML-*NPM1* patients with *DNMT3*Awt (blue) or *DNMT3A*mut (black) juxtaposed to all CETLAM-12 patients stratified according to ELN-17 risk classification



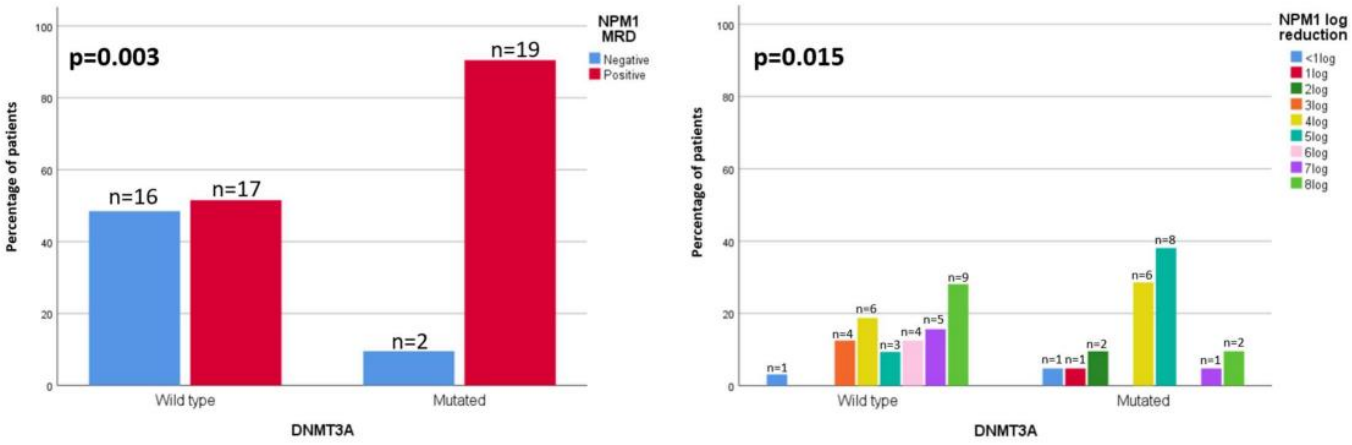
Supplemental figure 3: Overall survival of patients according to *DNMT3A* type of mutation. Comparison of outcome between patients with the R882 mutation or insertion/deletions, other missense mutations and the wild type cohort



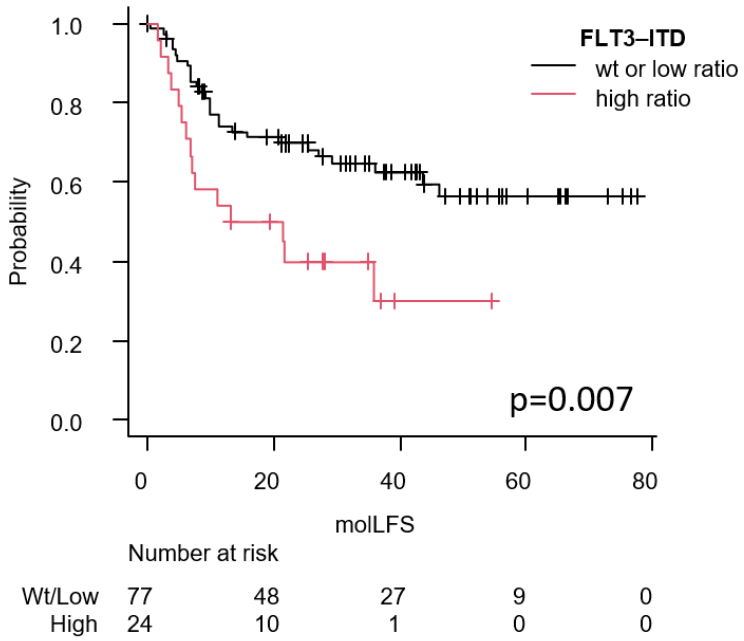
Supplemental figure 4: Survival impact of *DNMT3A* mutation in each *FLT3* subset. Overall survival (upper row) and leukemia free survival (lower row) of AML-*NPM1* patients with *FLT3*-ITD wild type, low ratio and high ratio according to their *DNMT3A* mutational status.



Supplemental figure 5: NPM1 measurable residual disease after consolidation-3: MRD response according to DNMT3A mutational status after the third consolidation by the percentage of patients with positive or negative MRD and log10 reduction.



Supplemental figure 6: Molecular leukemia free survival of AML-NPM1 patients divided by FLT3-ITD risk groups (wt: wild type, low: low allelic rato, high: high allelic ratio



4.2. Article 2

Survival improvement of patients with *FLT3* mutated acute myeloid leukemia: results from a prospective 9 years cohort

Guadalupe Oñate, Marta Pratcorona, Ana Garrido, Alicia Artigas-Baleri, Alex Bataller, Mar Tormo, Montserrat Arnan, Susana Vives, Rosa Coll, Olga Salamero, Ferran Vall-Llovera, Antònia Sampol, Antoni Garcia, Marta Cervera, Sara Garcia Avila, Joan Bargay, Xavier Ortín, Josep F. Nomdedeu, Jordi Esteve*, Jorge Sierra* and Spanish Cooperative Group for the Study and Treatment of Acute Leukemias and Myelodysplasias (CETLAM)

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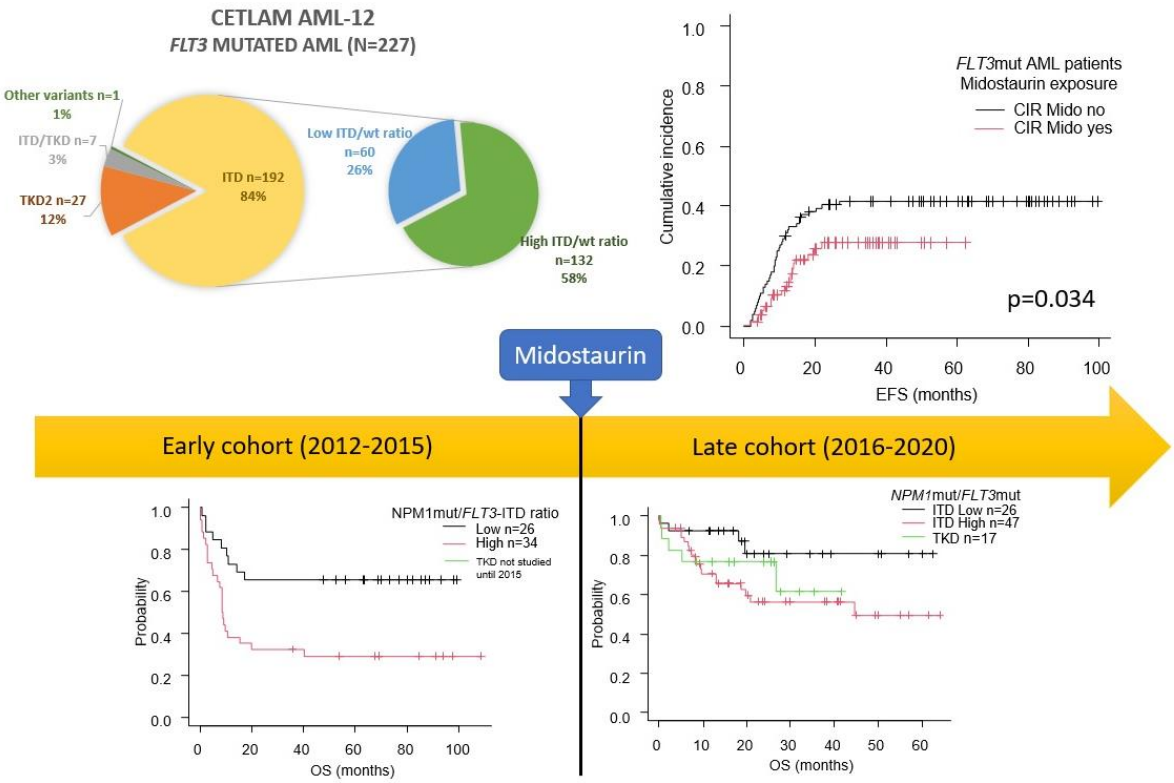
JCI (2021): 9.812

Visual abstract:



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ARTICLE OPEN

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Survival improvement of patients with *FLT3* mutated acute myeloid leukemia: results from a prospective 9 years cohort

Guadalupe Oñate[1], Marta Pratcorona[1], Ana Garrido[1], Alicia Artigas-Baleri[1], Alex Bataller[2], Mar Tormo[3], Montserrat Arnan[4], Susana Vives[5], Rosa Coll[6], Olga Salamero[7], Ferran Vall-Llovera[8], Antònia Sampol[9], Antoni Garcia[10], Marta Cervera[11], Sara Garcia Avila[12], Joan Bargay[13], Xavier Ortín[14], Josep F. Nomdedéu[1], Jordi Esteve[2,15], Jorge Sierra[1,15]✉ and Spanish Cooperative Group for the Study and Treatment of Acute Leukemias and Myelodysplasias (CETLAM)

Midostaurin added to intensive chemotherapy is the standard of care for acute myeloid leukemia (AML) with *FLT3* mutations (*FLT3*mut). We analyzed the impact of midostaurin in 227 *FLT3*mut-AML patients included in the AML-12 prospective trial for fit patients ≤70 years (#NCT04687098). Patients were divided into an early (2012–2015) and late (2016–2020) cohorts. They were uniformly treated except for the addition of midostaurin in 71% of late group patients. No differences were observed in response rates or the number of allotransplants between groups. Outcome was improved in the late period: 2-year relapse incidence decreased from 42% vs 29% in early vs late group ($p = 0.024$) and 2-year overall survival (OS) improved from 47% vs 61% ($p = 0.042$), respectively. The effect of midostaurin was evident in *NPM1*mut patients ($n = 151$), with 2-yr OS of 72% (exposed) vs 50% (naive) patients ($p = 0.011$) and mitigated *FLT3*-ITD allelic ratio prognostic value: 2-yr OS with midostaurin was 85% and 58% in low and high ratio patients ($p = 0.049$) vs 67% and 39% in naive patients ($p = 0.005$). In the wild-type *NPM1* subset ($n = 75$), we did not observe significant differences between both study periods. In conclusion, this study highlights the improved outcome of *FLT3*mut AML fit patients with the incorporation of midostaurin.

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INTRODUCTION

The treatment of patients with acute myeloid leukemia (AML) is rapidly evolving due to the advances in targeted therapy, risk-adapted protocols and measurable residual disease (MRD) guided decisions [1–4]. The implementation of molecular techniques with high sensitivity in everyday practice is now essential to accurately classify the different AML entities and to identify potential therapeutic targets in the AML cells. Therefore, a comprehensive molecular characterization of the disease is mandatory both at diagnosis and in the relapse and refractory setting for optimal treatment choice [5, 6].

FMS-like tyrosine kinase 3 (*FLT3*) is one of the most frequently mutated genes in AML with an incidence of around 30% and is generally associated with a negative outcome [7]. The most frequent mutations of *FLT3* are the internal tandem duplication (*FLT3*-ITD) near the juxta-membrane domain and point mutations of the TKD2 domain (*FLT3*-TKD), with an incidence of 22% and 8% respectively [8]. *FLT3* mutations (*FLT3*mut) result in the constitutive activation of the FLT3 receptor and the continuous transduction of pro-survival and proliferative signals via the RAS/MAPK, JAK/STAT5 and PI3K/AKT pathways [9].

The prognosis of patients harboring *FLT3*-ITD depends on several variables, such as the allelic ratio of the mutation, the presence of determinant co-mutations like *NPM1*, or the insertion site [10–14]. Regarding allelic ratio, several studies, including the National Comprehensive Cancer Network (NCCN) guidelines and the European LeukemiaNet 2017 classification (ELN-17) [15] emphasized its relevance in risk assessment. However, there is some controversy regarding the specific *FLT3*-ITD allelic threshold that can accurately divide high- and low-risk patients as well as concerns regarding the reproducibility of the technique. These are some of the reasons which led the experts to remove the modulating effect of *FLT3*-ITD allelic ratio in the latest prognostic classification of the ELN for AML (ELN-22) [16]. Anyhow, *FLT3*-ITD has in most instances a strong adverse impact and consequently, allogeneic hematopoietic cell transplantation (alloHCT) remains the recommended post-remission treatment in fit patients. Nonetheless, the indication of alloHCT in AML with *FLT3*mut

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might be redefined in the future given the potential benefit of *FLT3* inhibitors.

Midostaurin is a first-generation type 2 FLT3 inhibitor, with multi kinase inhibitory effect over various protein kinases such as FLT3, KIT, or PDGFR. The RATIFY phase III trial demonstrated a significant improvement in overall survival (OS) and event free survival (EFS) with the addition of midostaurin to standard 7 plus 3 chemotherapy in fit *FLT3*mut AML patients [17]. Based on this finding midostaurin was approved by the US Food and Drug Association and the European Medicines Agency in 2017 for all adult patients with AML with *FLT3* mutations.

A post-hoc analysis of the RATIFY trial demonstrated the benefit of midostaurin in reducing relapse risk in all ELN-17 prognostic categories [18]. However, the study was restricted to patients up to 59 years old. To date, only a few prospective phase II trials included patients older than 60 years who were intensively treated and also received midostaurin [19, 20]. These studies showed the feasibility of this combination in old fit patients as well as an overall outcome improvement compared to historical cohorts. Additionally, these studies also demonstrated the safety of midostaurin in the setting of post-alloHCT maintenance.

In 2016 the Spanish CETLAM group (Grupo cooperativo de estudio y tratamiento de las leucemias agudas y mielodisplasias) incorporated midostaurin to the therapy protocol for fit adults with AML and *FLT3* mutations. Patients were treated according the CETLAM AML-12 phase II trial (#NCT04687098). This included intensive chemotherapy (CT) induction followed by a consolidation approach within a risk-adapted post-remission strategy, with high-dose cytarabine or alloHCT according to the genetic risk at diagnosis and the persistence of MRD after treatment. This report focuses on the outcomes of *FLT3*mut patients up to 70 years before and after the introduction of midostaurin.

Two recent studies have analyzed the resulting effect of the incorporation of midostaurin [19, 21]. Up to the best of our knowledge, this constitutes the first study to address the issue in a patient population up to 70 treated with an homogenous chemotherapy backbone and a subsequent transplant decision based on a risk adapted criteria

SUBJECTS AND METHODS

Patients and samples

The series included patients with 18–70 years-old diagnosed with de novo AML and eligible for intensive chemotherapy within the CETLAM AML-12 phase II trial (#NCT04687098). Patients were treated at 15 academic centers in Spain between January 2012 and December 2020. Since 2016, patients with *FLT3*mut could receive midostaurin added to the therapy as part of the AML-12 trial. The trial was approved by the IRB boards at the participating institutions and by the health authorities and was conducted in accordance with the Declaration of Helsinki. Informed consent for both bone marrow analyses and treatment was obtained in all cases.

In order to analyze the impact of midostaurin on the outcome, we considered two study cohorts: "early cohort" (treated between 2012 and 2015) and "late cohort" (2016–2020). All patients received the same CT protocol except for the addition of midostaurin since 2016 in most *FLT3*mut patients. The criteria to indicate alloHCT in first CR (CR1) were unchanged throughout the study period.

Full protocol details are available at www.clinicaltrials.gov and are detailed in supplemental material. In brief, all patients enrolled in the AML-12 trial received induction chemotherapy with idarubicin and cytarabine. The definition of complete remission (CR), overall survival (OS), event-free survival (EFS) and cumulative incidence of relapse (CIR) followed the recommended ELN criteria [22]. Response assessment was performed after one cycle: patients with a partial response received a second induction cycle; if first complete remission with or without complete hematological recovery (CR or CRi) [23] was achieved, they proceeded with first consolidation with high dose cytarabine (HDAC) according to the classical CALGB scheme [24]. Risk stratification was based on ELN recommendations (ELN-2010 [23] until the introduction of ELN-2017 [15]) but patients with *NPM1*mut and *FLT3*-ITD low allelic ratio were considered favorable since the beginning of the protocol. Favorable-risk patients completed two more HDAC courses and continued with MRD monitoring, while intermediate or adverse patients were intended for alloHCT in CR1 after one or two HDAC consolidations. MRD was assessed in bone marrow samples after each treatment cycle by either multiparameter flow cytometry or molecular monitoring of *NPM1* transcripts (as described by Gorello et al.) and, in a few cases, of rearrangements *RUNX1::RUNX1T1* and *CBF::MYH11*.

Genomic analysis

All analysis were performed at diagnosis on bone marrow samples. Molecular testing of *NPM1* mutation was studied as previously described [25]. All patients were tested for *FLT3* internal tandem duplication (*FLT3*-ITD) [26] and classified according to its ITD/wt allelic ratio following previous recommendations (<0.5 low ratio, *FLT3*low; and ≥0.5 high ratio, *FLT3*high) [11, 15]. *FLT3*-TKD mutations were not routinely reported until 2015. Next-generation sequencing with a targeted panel of 42 genes was introduced in 2017 as part of the protocol diagnostic work-up allowing the detection of additional, less frequent *FLT3* mutations (Supplemental Fig. 2).

Statistical analysis

The analysis of the relationship between categorical variables was performed using the Chi-square test or the Fisher exact test. Differences between groups for continuous variables were studied by means of the independent-samples t-test or Mann-Whitney U. All tests were two sided and considered significant if $p < 0.05$. Follow-up duration was calculated with the inverted Kaplan-Meyer method [27, 28]. OS and EFS were studied with the Kaplan-Meier method, whereas cumulative incidences were calculated to estimate relapse risk and non-relapse mortality (NRM) considering death in remission and relapse as competitive end-points, respectively. Differences between groups were assessed with the log-rank test (OS, EFS) and the Gray test (CIR, NRM) and were considered significant when $p < 0.05$. Cox-proportional hazard regression was used for multivariable analysis. AlloHCT was analyzed as a time-dependent variable. All statistical analyses were performed with the SPSS software (Version 26, IBM, Armonk, NY, USA) and R statistics (Version 4.0.2, R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Characteristics of patients

FLT3 mutations were detected in 227 cases (25%) out of 906 patients included in the AML-12 protocol between 2012 and 2020, and constitute the study population. Median follow up is 42 months (95%CI 35–49). The early cohort included 94 patients whereas the late included 133. Characteristics of these patients, considering both time cohorts (2012–2015 and 2016–2020) are summarized in Table 1. Cytogenetics was available in 93% of cases. No significant differences were observed between the two study cohorts including the ELN prognostic category, prevalence of *NPM1* co-mutation or the proportion of patients harboring a *FLT3*-ITD with a high allelic ITD/wt ratio, with the exception of the virtual absence of TKD sole mutations in the early cohort. Since the analysis of *FLT3*-TKDs started in 2015, only 2 patients from the early cohort were identified compared to 25 from the late period cohort. Only one less frequent *FLT3* mutation (F594I) was detected in a patient with a complex karyotype.

Impact of midostaurin use

Seventy-one per cent (94/133) of patients from the late cohort received midostaurin at some point during frontline treatment (Fig. 1): 71 patients with an ITD (22 *FLT3*low and 49 *FLT3*high), 20 with TKD, and 3 with other *FLT3*mut. The onset of the treatment was during induction-1 in most patients (81%), although treatment start was deferred in the remaining patients due to an administrative gap between initial request and real availability of the drug during the initial period, which required compassionate request on a case-to-case basis. Fourteen patients of this group received midostaurin as maintenance, mainly ($n = 12$) due to a non-transplant allocation given a favorable genetic risk according to ELN-2017. Maintenance was administered in one

Table 1. Characteristics of patients treated according CETLAM AML-12 protocol and with an identified *FLT3* mutation.

CETLAM AML-12 patients < 70 years with *FLT3*mut (*n* = 227)			
	2012–2015 *n* = 94	**2016–2020 *n* = 133**	***P***
Female gender *n* (%)	52 (55)	75 (56)	0.9
Median age (range)	54 (21–70)	55 (20–70)	0.65
<60 years *n* (%)	65 (69)	87 (65)	
≥60 years *n* (%)	29 (31)	46 (35)	
ECOG 0–1 *n* (%)	77 (82)	102 (77)	0.3
Median WBC $\times 10^9$/L (range)	53 (1.6–314)	45 (0.42–395)	0.9
Median BM blasts % (range)	80% (21–100)	80% (21–100)	0.44
Cytogenetics prognostic category[&] *n* (%)			0.9
Favorable	4 (4)	4 (3)	
Intermediate	82 (88)	111 (83)	
Adverse	4 (4)	5 (4)	
No metaphases	4 (4)	13 (10)	
FLT3 mutations n (%)			
FLT3-ITD	92 (98)	100 (75)	0.54
Low ratio	33 (36)	27 (31)	—
High ratio	59 (64)	73 (69)	—
FLT3-TKD[#]	2 (2)	25 (19)	—
FLT3-ITD with TKD[#]	0	7 (5)	
Other	—	1 (1)	
Concomitant *NPM1*mut *n* (%)	61 (65)	90 (68)	0.7
ELN-17 prognostic categories			0.8
Favorable	30 (32%)	45 (34%)	
RUNX1::RUNX1T1	3	3	
CBFB::MYH11	1	1	
*NPM1*mut/*FLT3*low	25	25	
*NPM1*mut/*FLT3*-TKD	1	16	
Intermediate	38 (40%)	57 (43%)	
*NPM1*mut/*FLT3*high	34	47	
*NPM1*wt/*FLT3*low	3	5	
*NPM1*wt/*FLT3*-TKD	1	5	
Adverse	26 (28%)	31 (23%)	
*NPM1*wt/*FLT3*high	23	24	
Other*	3	7	
CR rate *n* (%)	73 (78)	109 (82)	0.7
AlloHCT *n* (%)	53 (56)	82 (62)	0.6
CR1	39 (41)	64 (48)	
CR2	6 (6)	9 (7)	
Active disease	5 (5)	7 (5)	
Unknown status	3 (5)	2 (2)	

AlloHCT allogeneic hematopoietic cell transplantation, *BM* bone marrow, *CR* complete remission, *ELN* European LeukemiaNet, *CR1* first complete remission, *CR2* second complete remission, *NA* not applicable, *OS* overall survival, *WBC* White blood count [&]Cytogenetic risk defined according to MRC/NCRI recommendations (Grimwade et al. Blood 2010) [#]TKD mutations were not routinely analyzed until 2015

*Two cases in the early period presented with t(6;9) while the other had a complex karyotype (CK), in the late period 2 patients had CK, 1 a t(6;9), 2 patients harbored mutated *TP53*, and 2 cases a mutated *RUNX1* with an absence of *NPM1*mut

patient with *NPM1*mut/*FLT3*high during the posttransplant period, whereas one unfavorable patient with *NPM1*wt/*FLT3*high was not eligible for alloHCT after chemotherapy, and started maintenance following consolidation-1.

Response rate after induction was similar in both time cohorts (CR/CRi rate 78% ($n = 73$) in the early group and 82% ($n = 109$) in the late period group; $p =$ ns). Treatment failure was related to refractory disease (12% in the early vs 11% in the late cohort, p=ns) or death in aplasia (11% vs 8%, respectively p=ns). An early relapse in the first 3.5 months (median time from CR to transplant) occurred in 9 patients between 2012 and 2015 and in 5 patients in the latter period 2016–2020 ($p = 0.089$). AlloHCT was performed in a similar proportion of patients in both time cohorts (in 56% ($n = 53$) and 62% ($n = 82$) of patients, respectively [$p =$ ns]). For patients achieving CR1, the alloHCT rate was 64% ($n = 47$) in the early group and 68% ($n = 74$) in the late group. In the overall cohort, MRD persistence following first consolidation was associated with higher relapse-risk (2 yr 48 ± 15% vs 28 ± 8% p = 0.021) and a trend towards worse survival (2 yr 57 ± 8% vs 70 ± 4% $p = 0.055$, Supplemental Fig. 3). However, we did not observe a significant association between MRD negativity and time period (73% MRD negative in early and late cohort) or midostaurin exposure (74% in both midostaurin naive and exposed patients).

Early cohort *FLT3*mut patients presented with a significantly higher CIR compared to the late cohort (2-year (2 yr) CIR of 42 ± 11% vs 29 ± 10% for early and late group, respectively; $p = 0.024$), without differences in NRM (2 yr NRM of 12% vs 13% respectively; $p = 0.8$; Fig. 2). This translated into an improved outcome of late cohort patients, regarding EFS (2-yr 37 ± 5% vs 50 ± 5% for early and late groups; $p = 0.021$) and OS (2-yr of 47 ± 5% vs 61 ± 5%, in each group, respectively; $p = 0.042$, Fig. 2).

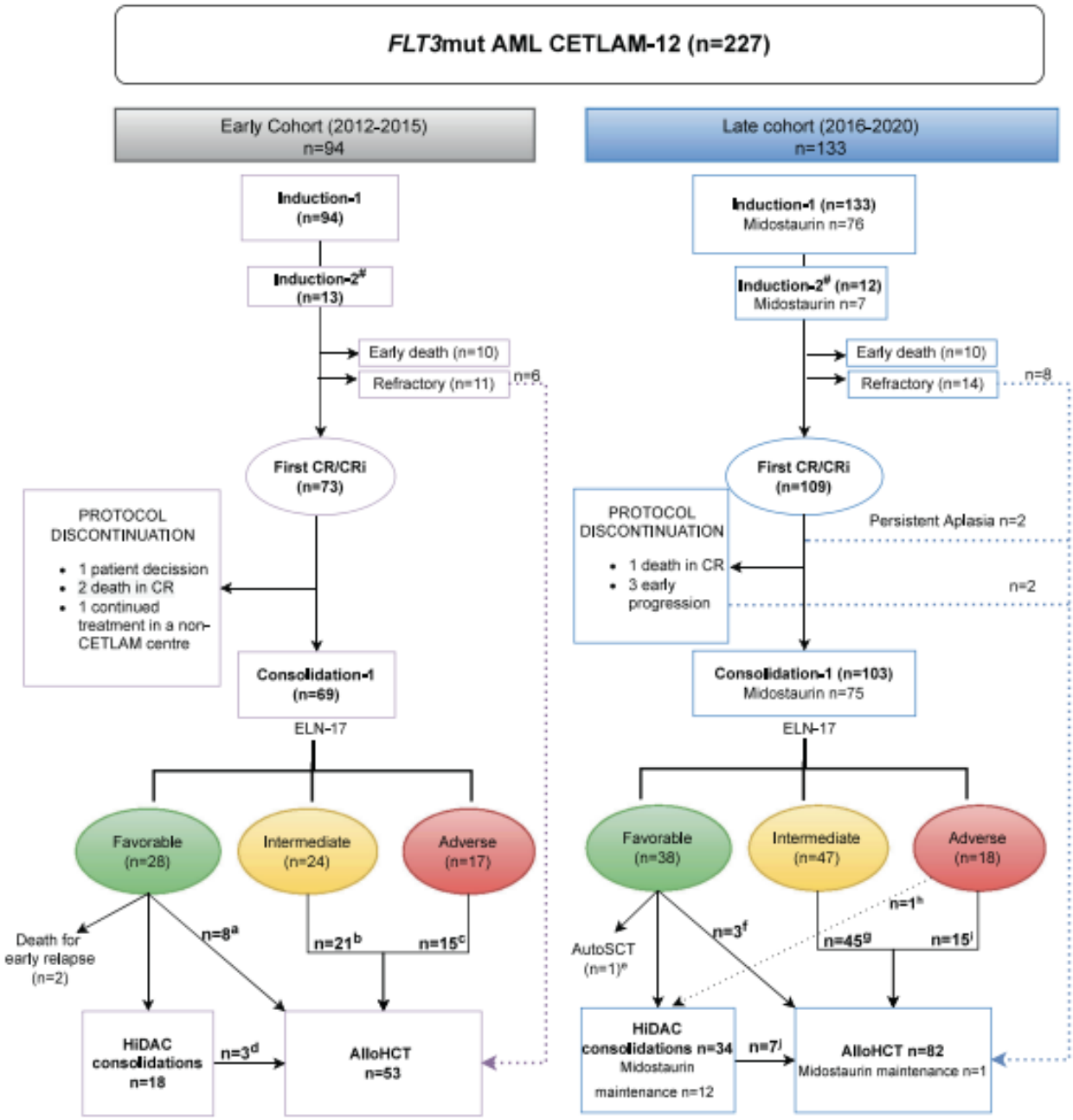
To establish the influence of different variables on survival, we performed univariate analysis for all *FLT3*mut patients, and found that leucocyte count (WBC) at diagnosis, treatment period, ELN-17 *FLT3* categories, and the presence of *NPM1*mut significantly impacted on OS (supplemental Table 1). The HR for OS of midostaurin administration was 0.62; 95%CI 0.41–0.94; $p = 0.024$. Thus, midostaurin led to improved survival in the whole cohort by decreasing relapse incidence (Fig. 3A) with a 2 yr RR of 40 ± 9% vs 28 ± 10% ($p = 0.034$) for naive and exposed patients, and a 2 yr OS of 49 ± 4% vs 65 ± 5% ($p = 0.023$) respectively. It is noteworthy that patients in the late cohort who did not receive midostaurin had very similar survival to those from the early cohort (Fig. 3B).

In a multivariate model including age, midostaurin use, WBC at diagnosis and ELN-17 prognostic categories, midostaurin maintained its independent prognostic value both for OS and EFS with a HR for OS of 0.55 (95%CI 0.36–0.85; $p = 0.007$), and for EFS of 0.51 (0.34–0.76; $p = 0.001$, Table 2), for patients exposed to the drug.

Impact of midostaurin in AML with *NPM1* mutation

A total of 151 patients harbored *NPM1*mut and *FLT3*mut simultaneously. There was an improved outcome of AML-*NPM1*mut patients who received midostaurin ($n = 65$), with a 2-yr EFS of 42 ± 5% vs 60 ± 7% for naive vs exposed patients ($p = 0.002$) and 2-yr OS of 50 ± 5% vs 72 ± 6% respectively ($p = 0.011$) (Fig. 4A). Moreover, the HR for OS of midostaurin exposure in the univariate analysis was 0.50 (95%CI 0.29–0.86, $p = 0.013$), a finding that was maintained in the multivariate analysis both for OS (HR 0.40 (0.22–0.72; $p = 0.002$) and EFS (HR 0.34 (0.20–0.59; $p < 0.001$, Table 2).

The allelic ratio of *FLT3*-ITD retained its prognostic value in both time cohorts (Fig. 4B). Thus, the 2-yr OS of patients treated during 2012 and 2015 was 65 ± 9% for *FLT3*low ($n = 26$) vs 32 ± 8% for



Only in patients who achieved partial response after induction-1
[a] 4 patients in CR1 MRD+, 1 in CR1 irrespective of *FLT3* ratio,1 due to persistant aplasia, 1 relapsed and achieved CR2
[b] The 3 patients did not receive alloHCT: 2 autologous transplant, 1 relapsed
[c] The 2 patients that were not transplanted: post-C1 relapse (n=1) or severe toxicities (n=1)
[d] 1 patient in CR1 molecular relapse, 2 relapsed and achieved CR2
[e] The patient who received autologous stem cell transplant (autoSCT) presented severe cerebritis with HiDAC.
[f] All cases for post-C1 MRD+
[g] 2 patients were not transplanted due to progresion and refractory disease
[h] 1 patient harboured *NPM1*mut/*FLT3*-TKD and *TP53*mut, was considered favorable and received consolidations and maintenance
[i] 1 patient relapsed and refractory disease, 1 patient proceeded directly to midostaurin maintenance due to complications during consolidation
[j] 2 patients presented molecular relapse, and 5 received alloHCT in CR2

Fig. 1 CONSORT diagram of patients in both time periods. AlloHCT: allogeneic stem cell transplant, AutoSCT: autologous stem cell transplant, C-1: first consolidation, CR1: first complete remission, CR2: second complete remission, HiDAC: high-dose cytarabine, MRD +: positive measurable residual disease.

*FLT3*high ($n = 34$; $p = 0.005$) whereas in the late period the adverse prognosis of *FLT3*-ITD was highly mitigated with a 2 yr OS of 81 ± 9% for *FLT3*low patients ($n = 26$) and 57 ± 8% for the *FLT3*high patients ($n = 47$) ($p = 0.033$). Similar findings were seen in terms of EFS (Supplemental Fig. 4) and when midostaurin exposure was directly contrasted (2-yr OS with midostaurin was

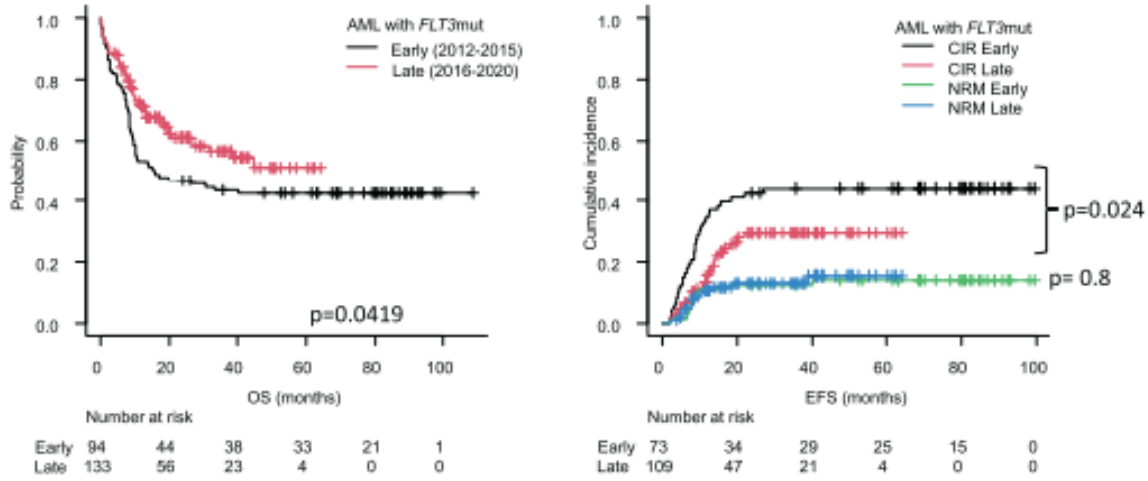


Fig. 2 Outcome of CETLAM AML-12 *FLT3*mut patients ($n = 227$), according to treatment period. CIR: cumulative incidence of relapse, EFS: event-free survival, NRM: non-relapse mortality, OS: overall survival.

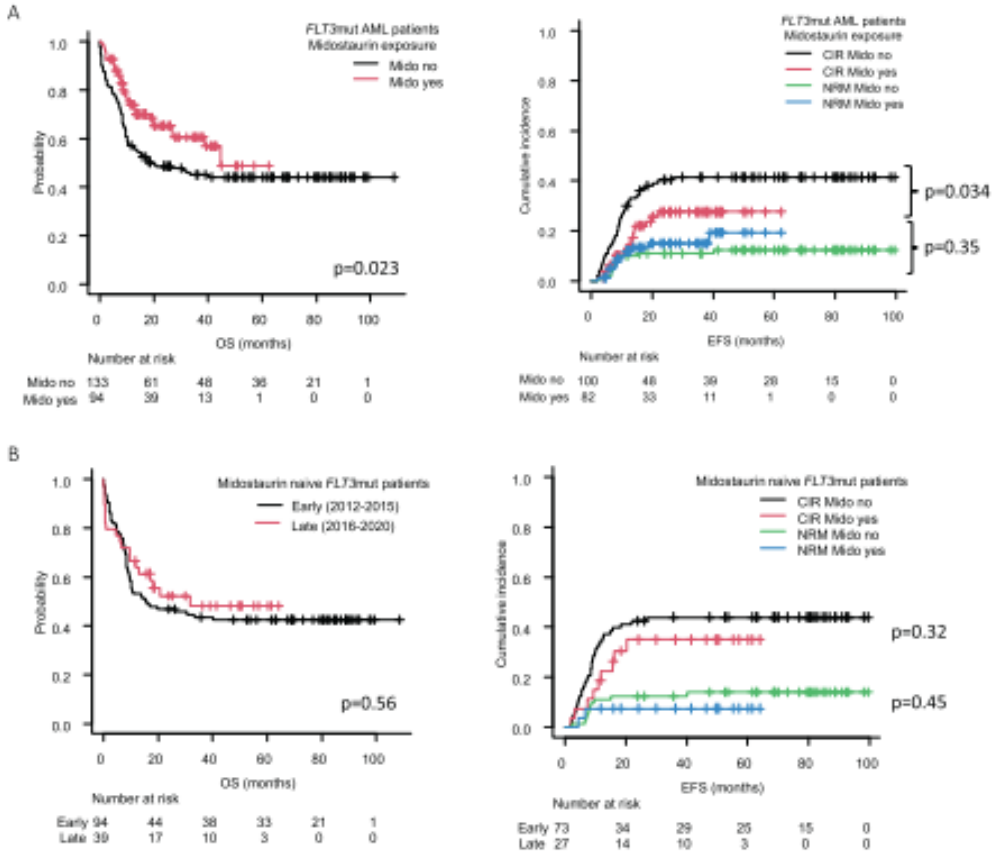


Fig. 3 Survival outcome of midostaurin use in AML-12 *FLT3*mut patients. A OS, CIR and NRM of the overall cohort according to midostaurin exposure. **B** OS, CIR, and NRM in both time periods among patients who did not receive midostaurin OS: overall survival, CIR: cumulative incidence or relapse, NRM: non-relapse mortality.

85% in low and 58% in high ratio patients ($p = 0.049$) vs 67% and 39% in naive patients; $p = 0.005$). On the other hand, *NPM1*mut/*FLT3*-TKD patients ($n = 18$) presented with a 2-yr OS of 72 ± 10%.It is worth mentioning that *NPM1*mut/*FLT3*low patients from the late cohort had a strikingly favorable outcome with a 2 yr OS of 81%, and more than half of those patients did not receive an alloHCT (64%, 16 out of 25 patients alive after induction). Among the 9 patients that did undergo alloHCT, 3 were performed due to positive MRD, 4 in CR2 status, 1 had active disease, and the disease status of the remaining patient was unknown (Fig. 5). Moreover, midostaurin was administered to 83% of patients from the late cohort that presented with non-high-risk disease (*NPM1*mut with *FLT3*low or TKD) who were never transplanted, and their 2-yr CIR was 5% in the late group ($n = 29$) vs 29% in the early group ($n = 14$, $p = 0.022$) with similar NRM (7%) in both cohorts (Supplemental Fig. 5).

Seventy-eight percent of *NPM1*mut/*FLT3*high patients (63 out of 81) received an alloHCT in the overall cohort, with 84% of them transplanted in CR1 (Fig. 5). We observed a significantly lower CIR of *NPM1*mut/*FLT3*high patients who received an alloHCT in the

Table 2. Multivariate Cox-proportional hazard regression in each *FLT3*mut subset.

	OS			EFS		
FLT3*mut*	**HR**	**95% CI**	***p***	**HR**	**95% CI**	***p***
WBC	1.002	1–1.005	0.025	1.003	1.001–1.005	0.003
Age <60 years	0.61	0.41–0.91	0.016	0.61	0.42–0.88	0.008
ELN-17 subcategories						
intermediate risk (vs fav)	2.36	1.38–4.04	0.002	2.21	1.38–3.55	0.001
adverse risk (vs. fav)	3.47	1.99–6.04	<0.001	3.19	1.95–5.23	<0.001
Midostaurin	0.55	0.36–0.85	0.007	0.51	0.34–0.76	0.001
*NPM1*mut *FLT3*mut						
WBC	1.002	0.999–1.005	0.25	1.003	1.000–1.006	0.027
Age<60 years	0.50	0.29–0.85	0.011	0.53	0.33–0.87	0.012
ELN-17 interm vs fav risk	2.39	1.34–4.24	0.003	2.13	1.28–3.53	0.003
Midostaurin	0.40	0.22–0.72	0.002	0.34	0.20–0.59	<0.001
*NPM1*wt *FLT3*mut						
WBC	1.005	1.002–1.009	0.006	1.005	1.002–1.009	0.002
Age < 60 years	0.73	0.38–1.43	0.4	0.64	0.35–1.17	0.15
ELN-17 subcategories						
intermediate risk (vs fav)	1.93	0.38–9.91	0.43	2.02	0.52–7.89	0.3
adverse risk (vs. fav)	3.65	0.86–15.5	0.08	3.12	0.94–10.4	0.063
Midostaurin	1.07	0.55–2.08	0.9	1.09	0.59–2.03	0.8

CI confidence interval, *EFS* event free survival, *HR* Hazard ratio, *OS* overall survival, *WBC* leucocyte count

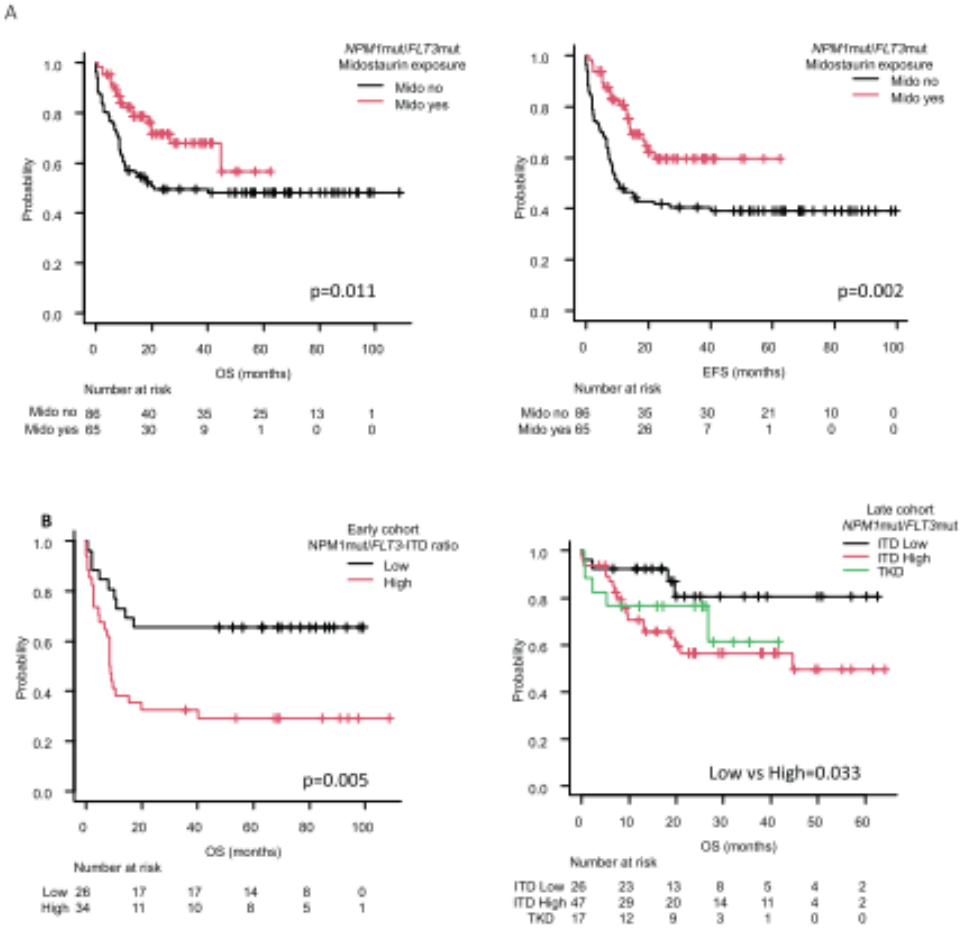


Fig. 4 Outcome of AML patients with FLT3 mutations and co-mutated NPM1. A OS and EFS of patients according to midostaurin exposure. **B** OS of each *NPM1*mut/*FLT3*mut subset in the early (left) and the late groups. *FLT3*-TKD mutations were not available in the early period.

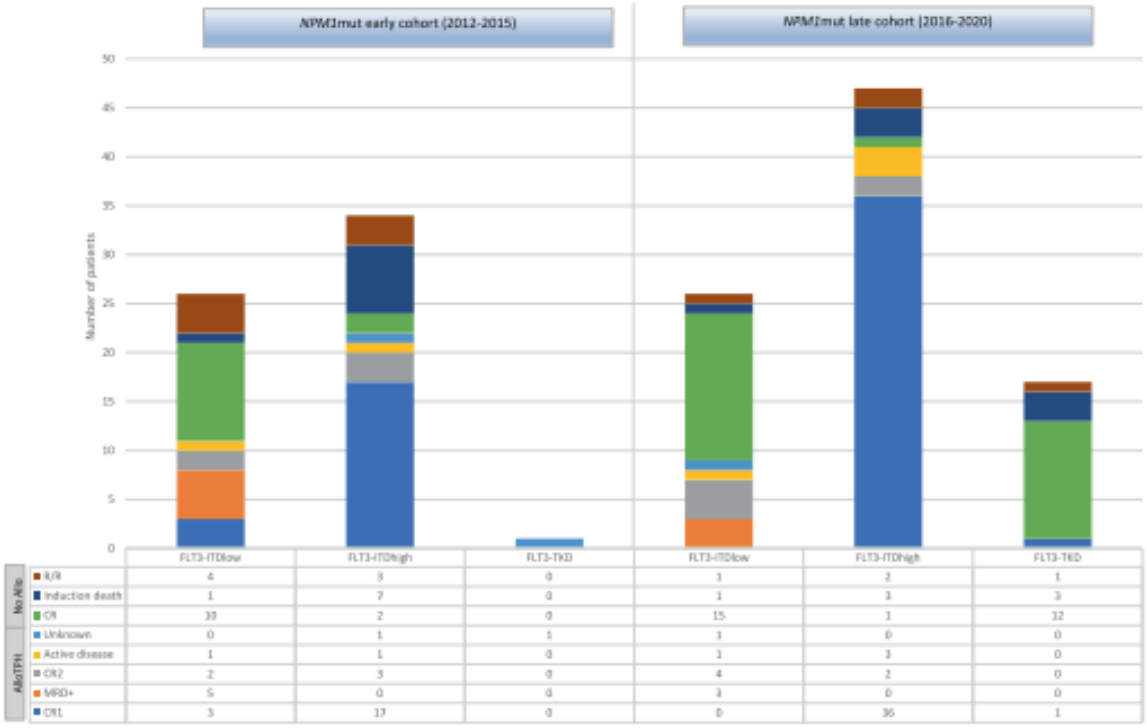


Fig. 5 Description of the disease status of *NPM1*mut/*FLT3*mut patients in each time period and *FLT3* subset. In allogeneic stem cell transplant (AlloHCT) recipients, pre-transplant evaluation is shown, in non-allotransplanted patients the last available evaluation is informed. CR complete remission (first: CR1, second: CR2), MRD + positive measurable residual disease, R/R relapse or refractory disease.

late group (71% of which had received midostaurin) in contrast to the early group (2-yr 55% vs 30%; $p = 0.044$) with no differences in NRM ($p = 0.2$; Supplemental Fig. 6).

Impact of midostaurin in AML with wild-type *NPM1*

Seventy-five patients presented with *FLT3*mut without mutation of *NPM1*, 33 and 42 from the early and late periods, respectively. The main clinical characteristics were balanced between cohorts (supplemental Table 2). There were no significant differences in karyotype distribution which was mostly of intermediate risk (76%) according to the Medical Research Council definition [29]. Nine percent of patients presented with CBF rearrangements, and *FLT3*-ITD allelic ratio was high in 78% of ITD patients in both cohorts. Also, 8 out of the 9 patients who presented *FLT3*-TKD mutations were from the late cohort and none of them were associated to a favorable karyotype.

Fifty-four patients (72%) achieved CR1 following induction and alloHCT was performed in 58% (19/33) and 74% (31/42) patients from the early and late cohort respectively ($p = 0.3$; Supplemental Fig. 7). Twenty-nine out of 42 late cohort patients received midostaurin (69%). Although the cohort size was limited, in the absence of *NPM1*mut we found no significant survival improvement with the use of midostaurin with a median EFS of 10 months for naive and exposed patients ($p = 0.9$) and a 2-yr OS of 48 ± 7% vs 51 ± 10% in the same groups respectively ($p = 0.9$, Supplemental Fig. 8), with a HR for OS (midostaurin) of 0.95 (95%CI 0.51–1.79; $p = 0.9$). A trend towards higher relapse rate was seen in the early group (2-yr CIR 54 ± 19% vs 33 ± 17% in the early and late groups; $p = 0.14$, Supplemental Fig. 9), but it did not translate into a significant survival improvement possibly due to the limited cohort size and a higher NRM of late cohort patients in this subgroup (NRM at 2 yr 8% vs 28% in the early and late groups, respectively $p = 0.10$). The global outcome of this molecular subgroup of patients remained poor throughout the protocol with a median EFS around 10 months in both groups, and a 2-yr OS of 49 ± 9% vs 48 ± 8% in the early and the late groups ($p = 0.9$, Supplemental Fig. 10). In the absence of *NPM1*mut, the small subset of non-ITD patients ($n = 10$: 9 TKDs and 1 other mutation) maintained their favorable outcome with a 2-yr OS of 73 ± 16%.

DISCUSSION

This study confirms the benefit of midostaurin in patients with AML and mutations of *FLT3* eligible for intensive chemotherapy, a subgroup that includes 30% of adults with this disease and is associated with adverse outcomes [30–32]. This beneficial effect was observed in patients with an age up to 70 years, treated following the same therapeutic protocol with a post-remission strategy adapted to genetic risk. Moreover, the effect of midostaurin was mostly attributed to a decrease in relapse risk and was confirmed in a multivariate analysis.

In our experience, the prognostic improvement was predominantly observed in patients with *NPM1* co-mutation. Of note, the outcome of patients both with *NPM1*mut/*FLT3*-ITD low ratio with an OS of 81% at two years, and *NPM1*mut with *FLT3*-TKD mutations (2-year OS 71%) was remarkably improved in the most recent period 2016–2020, two subgroups without an initial alloHCT intention in CR1. Overall, our experience is consistent with the results of the RATIFY trial that provided the most solid data on the benefit of adding midostaurin to intensive front-line chemotherapy in *FLT3*mut AML. This worldwide trial randomized 717 *FLT3*mut patients to receive midostaurin or placebo with induction and consolidation CT and showed an improvement in survival in the group with FLT3 inhibitor in the whole series as well as in all the molecular subgroups. A few studies have validated this finding; Larson et al. reported a post-hoc analysis of the RATIFY trial emphasizing the benefit of midostaurin in decreasing the cumulative incidence of relapse (HR 0.71 (95% CI, 0.54–0.93); $p = 0.01$). The trial and the analyses included patients up to 59 years old [18]. The AMLSG 16–10 phase 2 study, showed the positive impact of midostaurin in 198 younger and 86 older (>60 years) patients treated between 2012 and 2016 and compared the

outcomes with historical controls from 5 AMLSG trials between 1993 and 2008 [19, 20]. Recently, the MD Anderson group published a series showing the improvement in prognosis of patients with *FLT3*mut AML, mostly due to the implementation of targeted therapy with FLT3 inhibitors. In contrast to our study, they included patients mainly treated with sorafenib or quizartinib which are not commonly used as front-line therapy in this situation, and the report analyzed the addition of the FLT3 agents both in intensive and non-intensive combinations [21]. Differently from this, our study included only intensively treated patients and, as the AMLSG study, we analyzed patients up to the age of 70 years who were homogeneously treated with intensive CT and transplantation, the latter indicated based on risk assessment. It is important to emphasize that in our report 33% of the patients had an age between 60 and 70 years.

In the AML-12 trial the indication of alloHCT was defined according to risk at diagnosis and MRD evolution; in all intermediate and adverse patients the intention was to proceed to alloHCT unless major complications aroused during chemotherapy and in the favorable genetic category only patients in remission with persistent or reappearing MRD or those with cytological relapse were considered for alloHCT. All these interventional strategies did not allow, in our view, a fair assessment of the impact of alloHCT in the multivariable analysis. In fact, when we made an exploratory assessment of the impact of transplantation in the favorable ELN-17 subgroup (*NPM1*mut with *FLT3*low or TKD) and performed a Cox regression with transplantation as time-dependent covariate, a worse survival in transplant recipients was observed (HR for OS (Allo) 4.71 95% CI 1.31–16.95 $p = 0.018$) that we consider was attributable to the worse characteristics of transplanted cases (MRD positive or in relapse).

One of the limitations of our study is that we retrospectively compared two different study periods, and the favorable outcome of the late cohort might be influenced by other factors such as changes in alloHCT platforms or advances in support measures. However, during the period analyzed, neither chemotherapy strategies nor transplantation techniques substantially changed in our cooperative group and the only major advance was the introduction of midostaurin. Also, the outcomes of patients with AML that we treated with an identical protocol without *FLT3* mutations did not change between 2012 and 2020 (data not shown), and the OS improvement of *FLT3*mut patients was related to a decrease in their relapse risk. These facts reinforce that the improved outcome of *FLT3*mut-AML patients in recent years was due to the addition of this agent to the chemotherapy courses.

In accordance with previous CETLAM publications, we also performed molecular subgroup analyses. We, as the AMLSG, stratified patients according to the *FLT3*-ITD allelic ratio of 0.5. A prior study from our group revealed that this threshold had prognostic impact [11] and in a recent analysis we confirmed that this allelic ratio remained relevant regardless of the presence of *DNMT3A* co-mutation [4]. Of note, both studies analyzed *FLT3*mut patients treated before FLT3 inhibitors were available. Furthermore, a publication by Dohner et al. validating the ELN-17 classification in the RATIFY cohort showed that 0.5 was also the best discriminant value to define patients with different prognoses based on allelic ratio [12]. This differs from the original RATIFY trial that considered the 0.7 ratio cut-off. However, the limitations in the reproducibility of the technique to establish the ratio and the results from other investigators have led to the withdrawal of this aspect in the most recent ELN 2022 genetic classification of AML [16]. These ELN-2022 guidelines consider all *FLT3*-ITD patients as intermediate risk regardless of *NPM1* co-mutation or ITD allelic ratio and recommend alloHCT in CR1. The results from our study in patients with *NPM1*mut/*FLT3*-ITD low ratio challenges this recommendation; in our view, the results observed with consolidation CT only support continuing our current practice of delaying transplantation after a relapse, provided that there is no persistence of MRD. These patients had an outstanding OS since the introduction of midostaurin with a relapse incidence of less than 10% at 2 years. On the other hand, the results observed in the *NPM1*mut/*FLT3*-ITD high ratio who were candidates for alloHCT in CR1 also were significantly improved after midostaurin was introduced. In this regard, in this molecular category OS at 2 years was higher than 50% in the midostaurin era. Further analyses are needed to identify whether in a subgroup of these patients, allograft could be avoided or delayed to a later moment based on MRD absence and pattern of co-mutations. On the other hand, additional progress is needed in the *NPM1*wild/*FLT3*-ITD group, since midostaurin in our hands did not improve their outcome. This particular finding should be weighed carefully due to the limited size number. We analyzed a possible impact of midostaurin exposure in the pretransplant setting in *NPM1* mutated vs wild type and found that the median number of days in the first group was 25 vs 18 in the second. This difference, however, was not statistically significant. Finally, TKD mutations were only available in the late cohort, and therefore their outcome could not be comprehensively contrasted. Additional division of ITD/TKD subsets as well as the impact of other co-mutations resulted in too few patient in each subgroup to provide statistical significance in the current analysis and should be explored in larger studies.

We consider that studies as the one reported here are of value. Randomized clinical trials set to register novel agents in AML patients usually present strict inclusion criteria regarding performance status, organ functions and coexistence of infections at diagnosis, among others. Therefore, it is useful to confirm their results in less restrictive trials from academic groups such as ours that make much fewer limitations to inclusion concerning real-life high ECOG scores or active infections and accept adjustments of drugs for renal or liver abnormalities that would make the patients ineligible for trials promoted by corporate sponsors. The complementarity of registration trials, academic experiences and real data gives a more solid picture of the impact of new drugs to improve the prognosis of a rare and difficult disease such as AML.

In summary, this study highlights the positive change in prognosis of *FLT3*mut patients due to the association of CT and targeted therapy with midostaurin, in patients included in a prospective trial during 9 years comprising patients with an age up to 70 years and with a risk-adapted pre-defined post-remission alloHCT policy. Further research is needed in patients with AML and *FLT3*mut in the absence of *NPM1* mutation. It will also be of interest to know if the results reported here can be further improved with other FLT3 inhibitors.

DATA AVAILABILITY

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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AUTHOR CONTRIBUTIONS

GO and MP designed research, GO analyzed data and wrote the paper. JS and JE supervised research and wrote the paper; JFN and MP coordinated genomic analysis, and AA created the molecular supplemental tables and graphs, AG and AB performed data extraction from the cooperative data base, MA, SV, RC, MT, AS, MC, AG, FVLL, OS, SGA, XO, and JB provided the clinical data and all authors reviewed the final manuscript.

CONFLICT OF INTEREST

JS reports personal fees from AbbVie, Vyxeos, Gilead, CSL Behring, Astellas, and Gilead; grants and personal fees from Novartis and Daiichi-Sankyo; and grants from Amgen and Jazz Pharmaceuticals. JE reports consultancy-honoraria from Abbvie, Novartis, Astellas, Jazz Pharmaceuticals, BMS-Celgene, Pfizer, Daichii-Sankyo and research grants from Novartis and Jazz Pharmaceuticals. The remaining authors declare no competing financial interests.

ADDITIONAL INFORMATION

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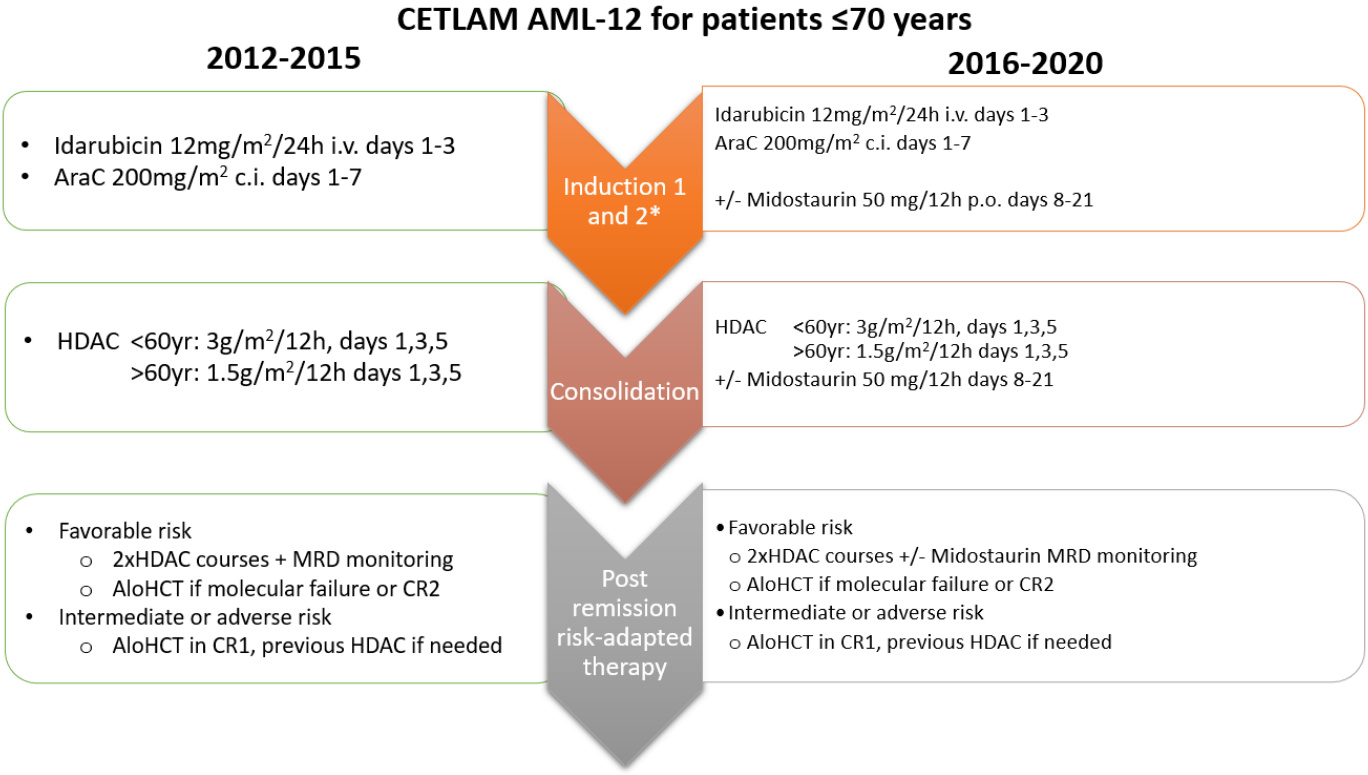
SUPPLEMENTAL MATERIAL

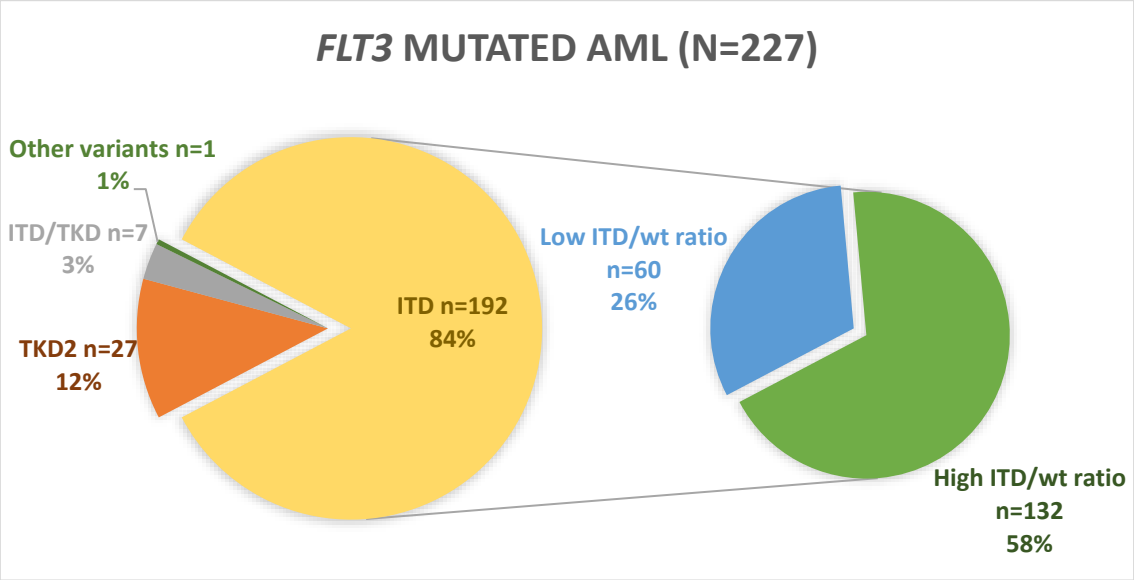
Oñate et al. Survival improvement of patients with FLT3 mutated acute myeloid leukemia: resulta from a prospective 9 years cohort.

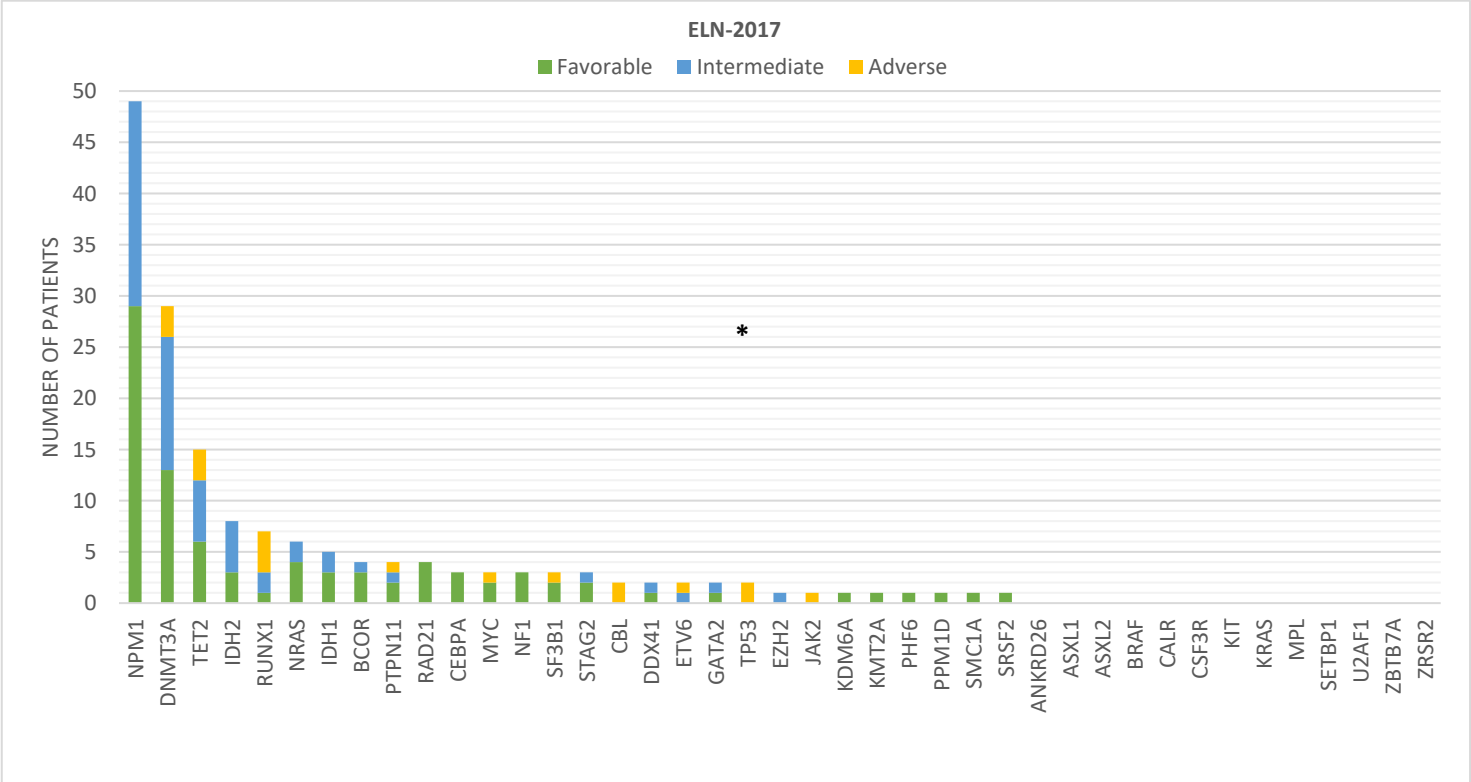
Supplemental Figure 1: CETLAM AML-12 intensive protocol algorithm.



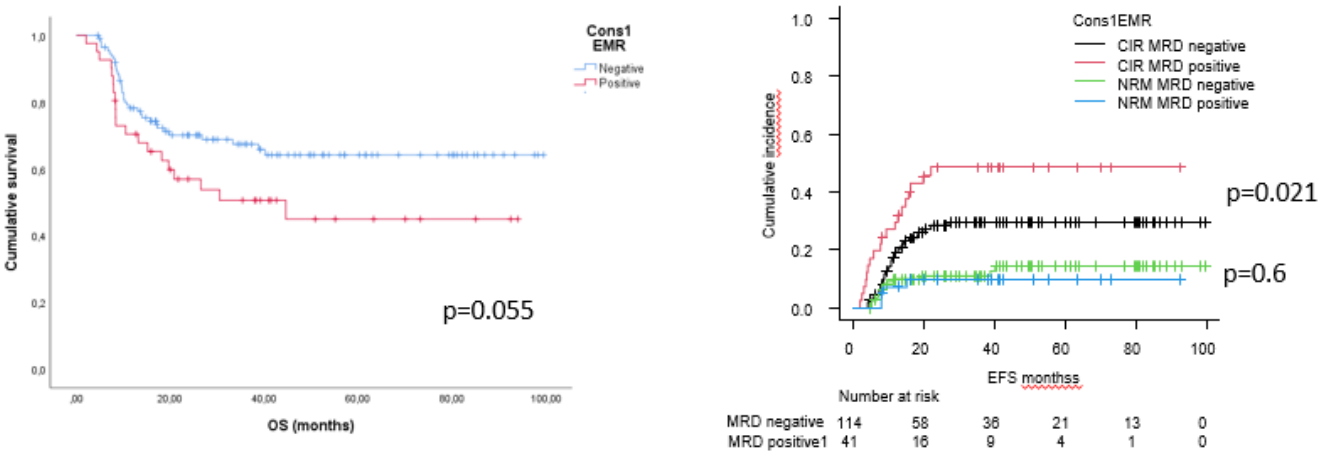
*Induction-2 only administered in case of partial response. AlloHCT: allogeneic stem cell transplant, CR1: first complete response, CR2: second complete response, HDAC: High dose cytarabine, MRD: Measurable residual disease, Yr: years

Supplemental figure 2: *FLT3* mutation distribution in the whole cohort (upper figure) and co-mutational pattern according to next generation sequencing results and ELN-17 categories. NGS was only included in the protocol from 2017 and was therefore performed in 67 patients from the late period (lower figure).





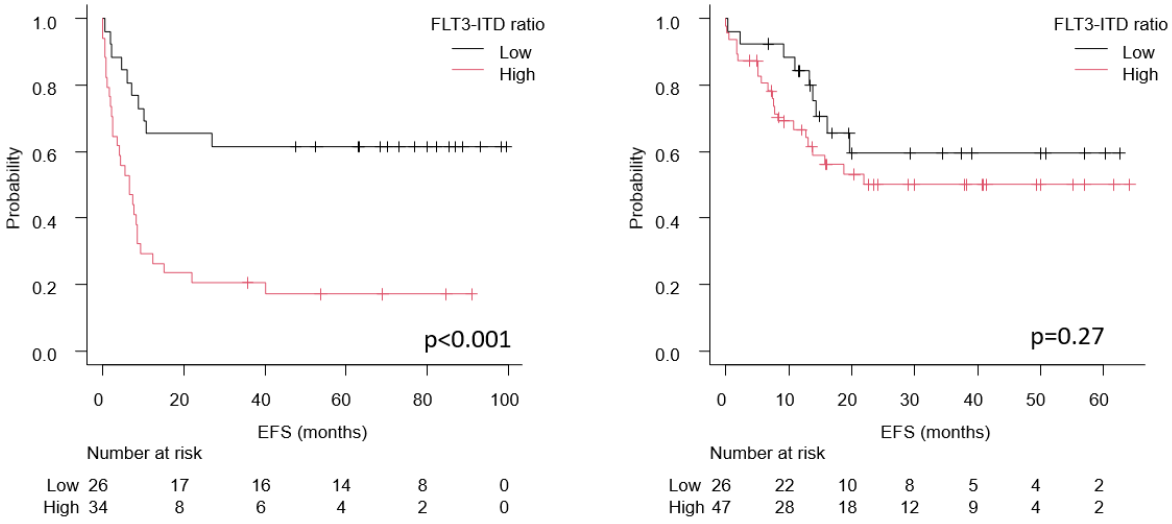
Supplemental Figure 3: Impact of MRD persistence after consolidation-1 in the overall cohort of *FLT3*mut patients by overall survival and cumulative incidence of relapse



Supplemental Table 1: Univariate Cox-regression for OS of all *FLT3*mut patients

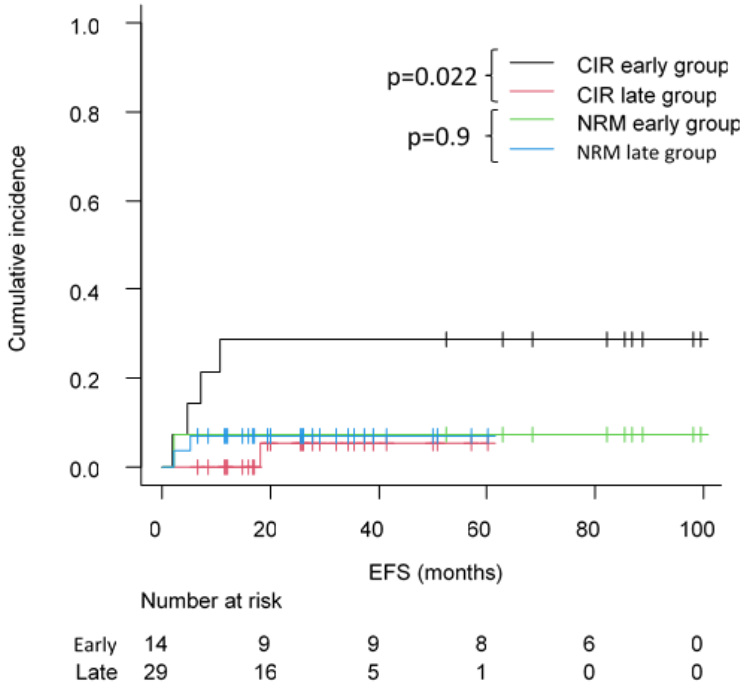
	HR for OS	95,0% CI		p
		Inferior	Superior	
Gender (male)	1.18	0.81	1.72	0.4
Age<60	0.68	0.46	1.005	0.053
Treatment Period Early	1.49	1.01	2.18	0.043
ELN-17 intermediate vs fav	2.51	1.48	4.27	0.001
adverse vs fav	3.6	2.07	6.26	<0.001
WBC at diagnosis	1.003	1.001	1.005	0.015
Bone marrow Blasts	1.001	0.991	1.010	0.9
***NPM1*mut**	0.67	0.46	0.99	0.046
FLT3-ITD high ratio	2.71	1.64	4.49	<0.001
Midostaurin	0.62	0,41	0,94	0.024
Post-induction result (refractory)	3.27	1.92	5.58	<0.001
Nº cycle to CR (1)	0.72	0.36	1.45	0.4

Supplemental Figure 4: Event free survival of early and late *NPM1*mut patients according to *FLT3*-ITD allelic ratio

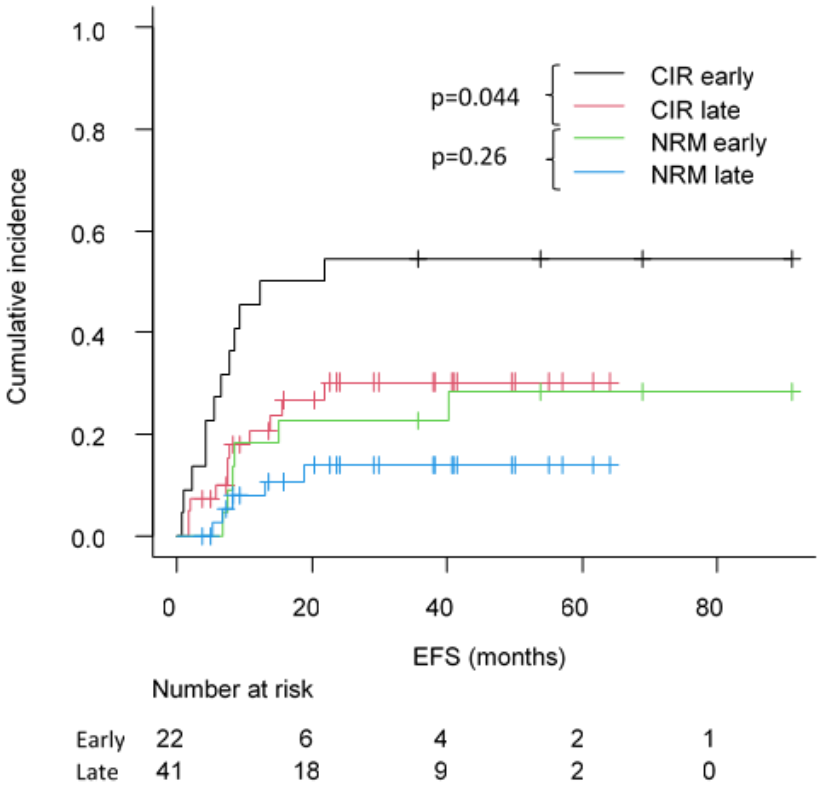


EARLY COHORT **LATE COHORT**

Supplemental figure 5: Cumulative incidence of relapse and non-relapse mortality of non-transplanted *NPM1*mut *FLT3*-ITD low ratio patients in each time period



Supplemental figure 6: CIR of allotransplanted patients with *NPM1*mut and *FLT3*-ITD high ratio in each time period.



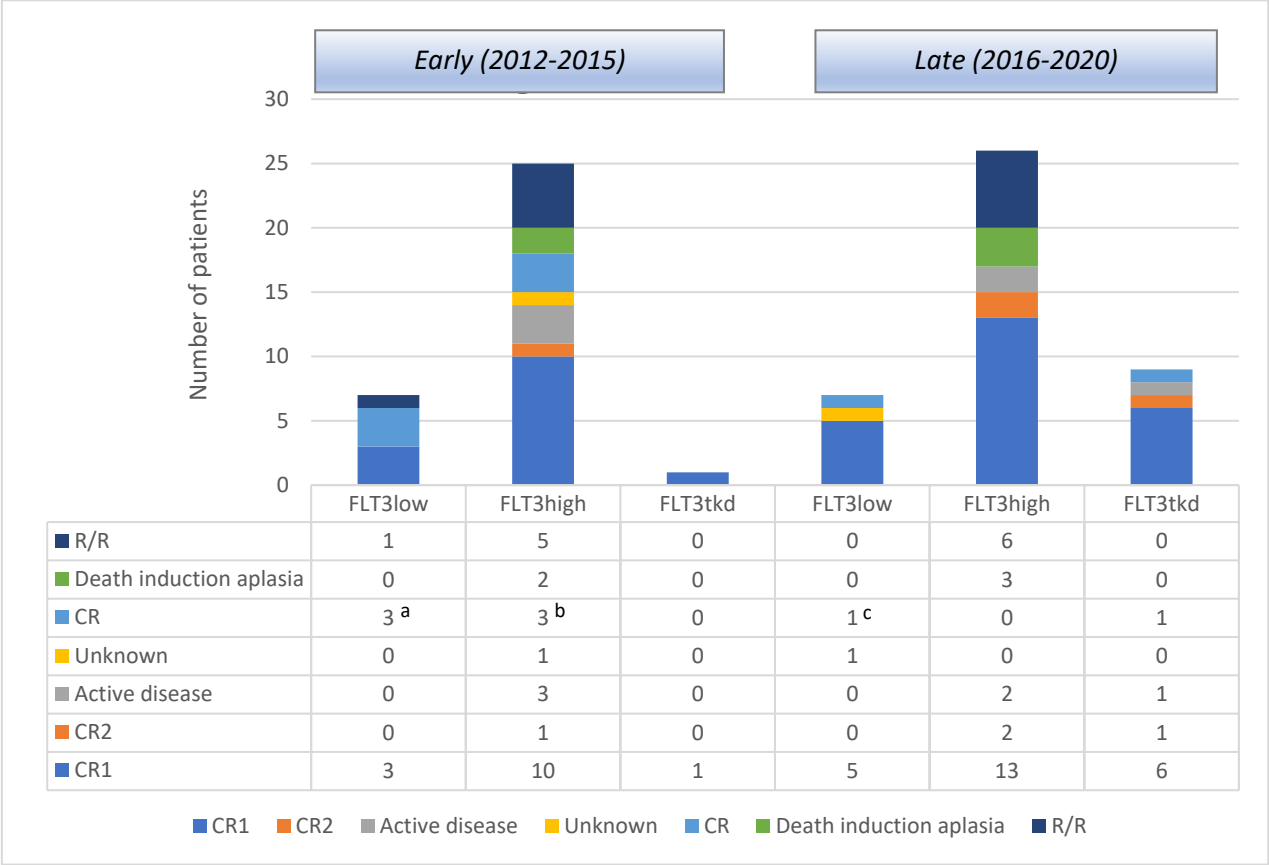
Supplemental Table 2: Patient characteristics of CETLAM AML-12 *NPM1*wt/*FLT3*mut patients

	2012-2015 (n=33)	**2016-2020 (n=42)**	**p**
Female gender n (%)	20 (61)	24 (57)	0.8
Median Age (range)	53 (21-69)	52 (22-69)	0.6
Median WBC x10^9/L (range)	42 (2-313)	22 (0.4-304)	0.08
Median % BM blasts (range)	82 (30-96)	75 (21-98)	0.1
Cytogenetics (MRC 2010), n(%)			0.9
Favourable	3 (9)	4 (9)	
Intermediate	26 (79)	31 (74)	
Adverse	3 (9)	5 (12)	
No metaphases	1 (3)	2 (5)	
***FLT3*-ITD n (%)**	32 (97)	33 (79)	0.9
Low ratio n	7	7	
High ratio n	25	26	
***FLT3*-TKD$^{\#}$ n (%)**	1 (3)	8 (19)	-
***FLT3*-other n (%)**	-	1 (2)	
CR after induction n (%)	24 (73)	30 (71)	0.9
Nº cycles to CR (1) n (%)	19 (79)	27 (90)	0.3
AlloHCT n (%)	19 (58)	31 (74)	0.3

BM: bone marrow, CR1: first complete remission, NA: not applicable, WBC: leucocyte count

TKD mutations detected only from late 2015

Supplemental Figure 7: Outcome distribution at last follow-up in *FLT3*mut/*NPM1*wt patients.



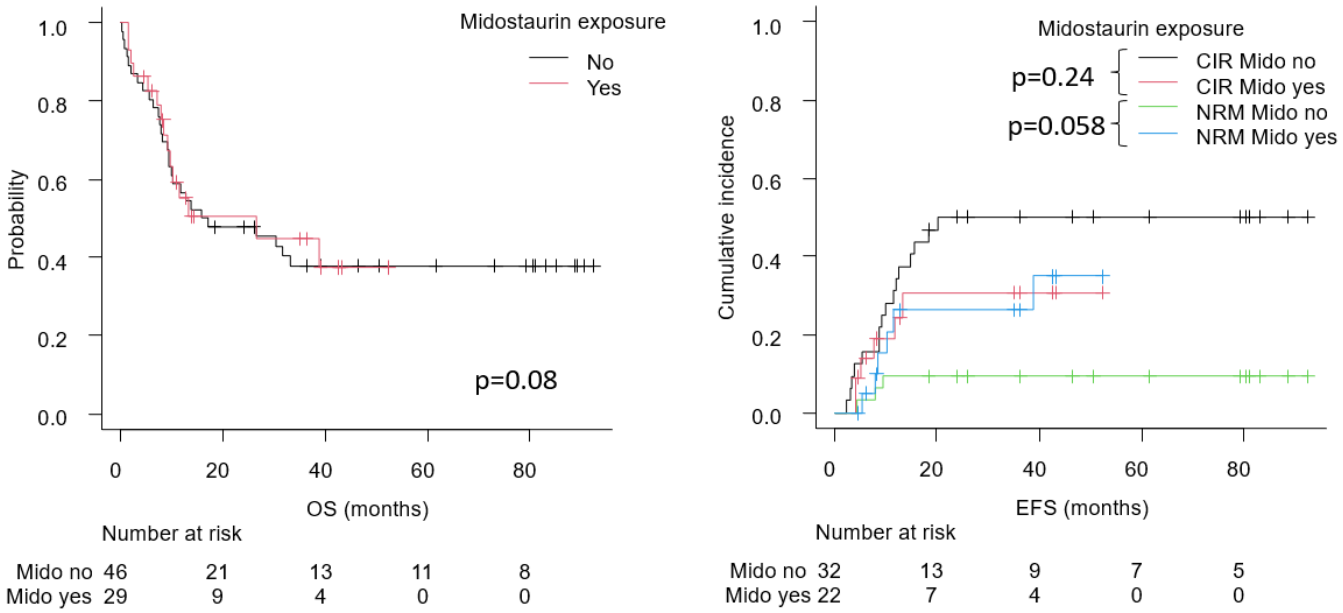
	FLT3low	FLT3high	FLT3tkd	FLT3low	FLT3high	FLT3tkd
R/R	1	5	0	0	6	0
Death induction aplasia	0	2	0	0	3	0
CR	3 [a]	3 [b]	0	1 [c]	0	1
Unknown	0	1	0	1	0	0
Active disease	0	3	0	0	2	1
CR2	0	1	0	0	2	1
CR1	3	10	1	5	13	6

[a]2 patients harboured CBF rearrengements (1 inv(16) and 1 t(8;21)), 1 patients received autologous transplant per center decission

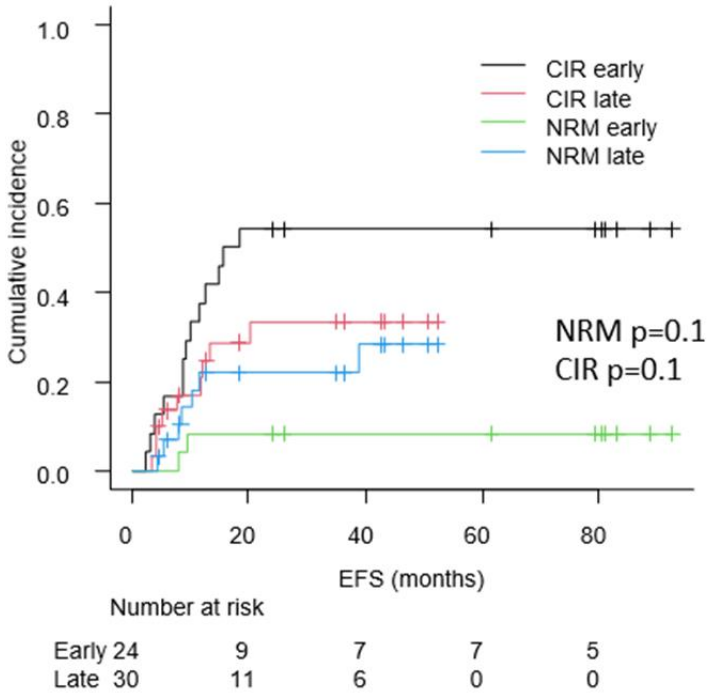
[b]2 patients with t(8;21)

[c]1 patient with inv(16)

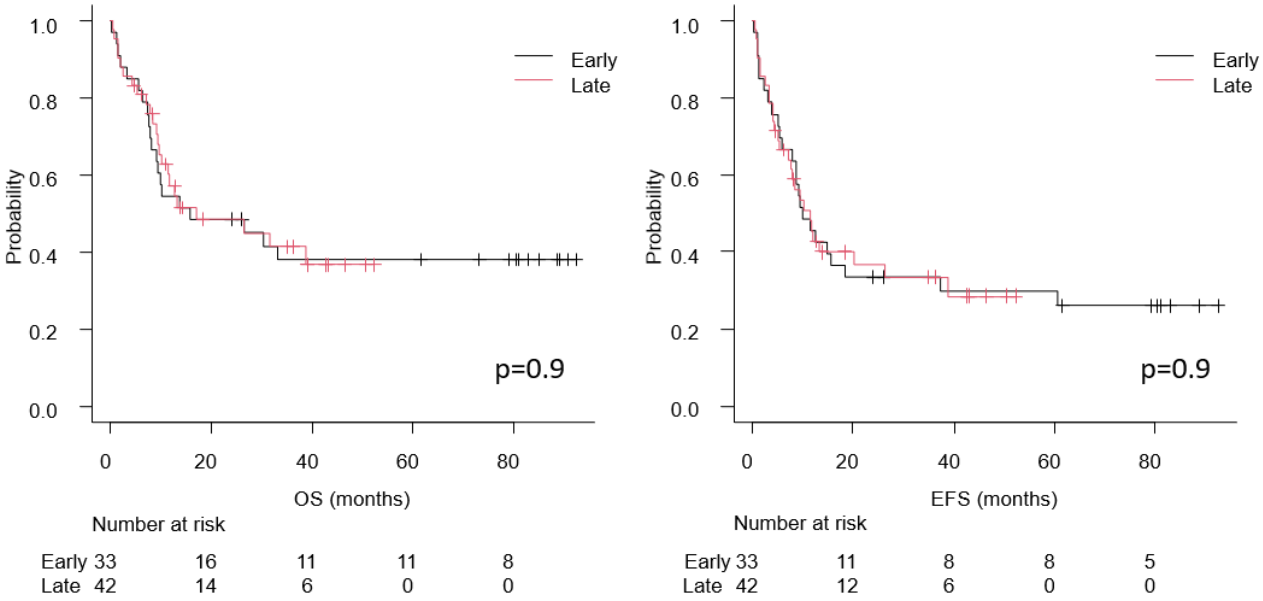
Supplemental Figure 8: Midostaurin effect in the subset of *FLT3*mut patients without *NPM1* mutation



Supplemental figure 9: Risk of relapse of *FLT3*mut/*NPM1*wt patients. CIR: cumulative incidence of relapse, NRM: non-relapse mortality



Supplemental figure 10: OS and EFS of *FLT3*mut/*NPM1*wt patients



5. OVERALL SUMMARY OF RESULTS

This section will itemize results of the research carried out in this thesis according to the initially set objectives. The first objective of this doctoral thesis was to study the impact of *DNMT3A* mutations in newly diagnosed AML patients that harbored *NPM1*mut, with or without a co-mutated *FLT3*-ITD. In the first article, we analyzed how the triple combination of mutations in *NPM1, FLT3,* and *DNMT3A* (the three most frequent mutated genes in adult AML) modified the outcome of AML patients. The rationale for this study is that AML with *NPM1*mut represents a separate category that affects 30% of adult patients with AML, with a prognosis known to be modulated by *FLT3* mutational status. Although *DNMT3A* is highly associated with *NPM1*mut, it is also associated with age-related clonal hematopoiesis and its prognosis in AML remains controversial.

For this purpose, we retrospectively analyzed data from 164 patients with AML and *NPM1*mut treated within the CETLAM Spanish cooperative group between 2003 and 2017 with the AML-03 (n=48) or AML-12 (n=116) intensive protocols. Briefly, patients received of intensive chemotherapy with one or two cycles of induction based on idarubicin and cytarabine (3+7), and one cycle of high-dose cytarabine (HiDAC). Subsequently, patients were stratified to receive post-remission consolidation either with alloHCT in intermediate or adverse-risk patients or two more cycles of HiDAC in favorable-risk patients. In the latter group, patients were followed with strict MRD monitoring and were only considered for alloHCT if a molecular or a morphological relapse were detected.

We found *DNMT3A* mutations (*DNMT3A*mut) in 48% (79/116) of patients. Clinical characteristics at diagnosis were comparable between patients with and without the mutation, except for a higher leucocyte count in the mutated group. Interestingly, the distribution of *FLT3*-ITD subsets (wild type/low ratio/high ratio) was independent of *DNMT3A* mutations.

With a median follow-up of 30 months, the presence of a mutated *DNMT3A* did not significantly modify the prognosis of the overall cohort neither for overall survival (5-yr OS 62% vs. 56% for *DNMT3A*wt vs. mut; p=0.2), leukemia-free survival (5-yr LFS 65% vs. 54%; p=0.1) or relapse-rate (5-yr CIR 22% vs. 31%; p=0.2).

In this study, *FLT3*-ITD allelic ratio validated its prognostic impact with similar survival and relapse risk in patients with either a wild-type *FLT3*-ITD or a *FLT3*low. Thus the 5-yr OS was of 67% and 62% for *FLT3*wt and *FLT3*low, while 5-yr CIR was 18% and 16% in the same groups. A significantly worse prognosis for high ratio patients (*FLT3*high) was observed with a 5-yr OS of 40% and a 5-yr CIR of 41%.

Then, we explored how the combination of both mutations modified the prognosis of *NPM1*mut leukemia patients. In the *NPM1*mut/*DNMT3A*mut cohort, the outcome of patients without *FLT3*-ITD was similar to *FLT3*low, with 5-yr OS and LFS around 60%, a 5-yr CIR around 25%, without statistical differences between groups. On the contrary, in this subset of patients, the presence of a *FLT3*high associated a highly adverse outcome with a 5-yr OS and LFS of 28% and 20%, respectively, and a 5-yr CIR of 48%. On the contrary, in *NPM1*mut patients without mutations in *DNMT3A* the deleterious effect of *FLT3*high ratio was mitigated (5-yr OS 47%, LFS 52% and CIR 33%), while the outcome between *FLT3* wild type and low ratio patients remained similar (5-yr 66% in both groups and 5-yr CIR 23% in *FLT3*tw and 29% in *FLT3*mut, p=0.4).

The second objective of this thesis was to analyze whether *DNMT3A* mutations modulated molecular MRD clearance or increased molecular relapses. This was considered a relevant point, not only for the validated prognostic impact of MRD in *NPM1*mut AML patients, but also because molecular relapse in the absence of cytological disease was in our protocol a criterion to perform alloHCT in first complete remission in otherwise ELN-17 favorable patients.

Among the 94 patients with available molecular MRD data, no differences were detected in the CR rate depending on the presence of *DNMT3A*mut but molecular MRD kinetics differed according to *DNMT3A* mutational status. Patients with *DNMT3A*mut showed a higher number of absolute *NPM1*mut transcripts after induction (p=0.012) and consolidation-1 (p<0.001). Similar results were also seen after consolidations 2 and 3. Of note, none of the *DNMT3A*mut patients achieved MRD negativity after consolidation-1 compared to 32% of *DNMT3A*wt (p=0.001), and responses were clearly deeper in patients without the mutation, with 77% of cases achieving ≥4log reduction vs. 46% in the *DNMT3A* mutated group (p=0.033).

Interestingly, in the triple mutated group (*NPM1*mut*/DNMT3A*mut*/FLT3*-ITD), all patients remained MRD positive after induction, C1 and C2 regardless of *FLT3* allelic ratio.

Finally, among 85 cases included in the AML-12 protocol not initially considered for alloHCT in CR1 (63 *NPM1*mut/*FLT3*wt, 22 *NPM1*mut/*FLT3*low), a trend towards a worse long-term sustained molecular CR was observed in *DNMT3A*mut patients compared to *DNMT3A*wt cases (molecular LFS 50 vs. 63% p=0.054).

As exposed in the introduction of this thesis, several advances in target therapy have been recently achieved for AML patients. One of the most studied targets is FLT3, a tyrosine kinase protein for which several inhibitors have been developed with exciting results published from various phase II and III trials. The first FLT3 inhibitor was midostaurin, and results from the RATIFY trial phase III randomized, placebo-controlled trial led to its approval by the FDA and the European Medicines Agency in 2017. Although a few recent studies have explored the real-life benefit of using TK inhibitors and the consequent change of prognosis of *FLT3*mut patients, they either included heterogeneously treated patients (fit and unfit), jointly analyzed different FLT3 inhibitors or did not consider patients older than 60 years.

The CETLAM AML-12 trial ran from 2012 to 2022 and included fit AML patients from 18 to 70 years. Patients were treated homogeneously throughout the protocol with the same chemotherapy regimen and alloHCT criteria, and it was only modified to include midostaurin from 2017 in most *FLT3*mut patients. For this reason, the third objective of this thesis was to assess the change in prognosis of patients with AML and *FLT3* mutations before and after the incorporation of midostaurin in an evenly treated cohort of patients eligible for intensive chemotherapy.

In the second article, we report the results of 227 *FLT3*mut patients from the AML-12 protocol. In order to explore the changes resulting from the introduction of midostaurin we divided patients into an “early cohort” from 2012 to 2015 (n=94) and a “late cohort” from 2016 to 2020 (n=133). All main clinical characteristics were equivalent between cohorts, specially ELN-17 distribution, *NPM1*mut frequency, and *FLT3*-ITD allelic ratio distribution. Seventy-one percent of the late cohort patients (94/133) received midostaurin at some point during frontline treatment, and 14 patients received it as

maintenance, mainly (n=12) favorable patients that did not undergo alloHCT in first CR.

Responses to induction treatment were similar in both cohorts: around 80% of complete responses, approximately 10% of refractory patients, and 10% of deaths in aplasia in both groups. AlloHCT in CR1 was performed in 64% and 68% of the early and late cohort patients. With a median follow-up of 42 months, late cohort patients presented with significantly improved OS, attributed to an ameliorated EFS and a lower CIR. In early vs. late cohorts, the two-year (2-yr) OS was 47% vs. 61% (p=0.042), with a 2yr-EFS of 37% vs. 50% (p=0.021) and a 2-yr CIR of 42% vs. 29% (p=0.024).

To discern the most significant variables associated with this survival advantage, we performed univariate analysis on the whole cohort of *FLT3*mut patients. We observed that WBC at diagnosis (p=0.015), treatment period (p=0.043), and ELN-17 *FLT3* categories (p=0.001) were the variables with a highest impact on OS. The univariate hazard ratio (HR) for OS of midostaurin exposure was 0.62 (95% confidence interval (CI) 0.41-0.94 p=0.024).

Thus, we further validated the impact of midostaurin in a multivariate model including age, WBC, and ELN-17 prognostic categories, and observed a sustained benefit of midostaurin both for OS (HR 0.55, 95%CI 0.36-0.85; p=0.007) and EFS (HR 0.51, 95%0.34-0.94; p=0.001). These results were corroborated with Kaplan-Meyer analysis and log-rank tests. Midostaurin improved survival by decreasing relapse risk with a 2-yr CIR 40% vs. 28% for naïve and exposed patients (p=0.034) and improved 2-yr OS (49% vs. 65% respectively; p=0.023). Of note, patients in the late cohort that did not receive the drug presented with equivalent survival as those from the early cohort (p=0.56).

Once the impact of midostaurin among *FLT3*mut patients was stablished, and considering that our group had repeatedly validated the value of *FLT3*-ITD allelic ratio, the last objective of this thesis was to analyze how midostaurin affected the different molecular subsets of *FLT3*mut AML.

First, we focused on patients that harbored co-mutations in *FLT3* and *NPM1* (n=151) and observed even higher differences between naïve and exposed to midostaurin patients with a 2-yr EFS of 49% vs. 65% (p=0.023) and a 2-yr OS of 49% vs. 65%

respectively (p=0.023). Multivariate HR for OS of midostaurin in this subset was 0.4 (95% CI 0.22-0.72; p=0.002).

Second, we evaluated the value of the *FLT3*-ITD allelic ratio. Early cohort patients showed survival rates for each allelic subgroup that mirrored our previous publications with 2yr OS for *FLT3*low and high of 65% and 32% (p=0.005). However, in the late cohort both subgroups showed a striking improvement of OS and EFS with a 2-yr OS of 81% for *FLT3*low patients (n=26) and 57% for *FLT3*high (n=47; p=0.033). Worth mentioning, 64% of late cohort patients with *NPM1*mut/*FLT3*low were never transplanted, and only nine patients received an alloHCT for different reasons (4 patients in CR2, 3 for MRD+, 1 active disease, and 1 unknown). Meanwhile, most of the *FLT3*high patients were transplanted as per protocol (78%; 83% in CR1), but a higher relapse rate was observed in the early group compared to the late group in which 71% of patients had been exposed to midostaurin (2-yr CIR 30% vs. 55% p=0.044).

We also analyzed the direct effect of midostaurin exposure, which was significantly beneficial for both allelic subgroups: 2-yr OS with midostaurin was 85% in low and 58% in high ratio patients (p=0.049) vs. 67% and 39% in naïve patients (p=0.005). Moreover, in the specific subgroup ELN17-favorable (*NPM1*mut with *FLT3*low or TKD) who never received an alloHCT, the 2-yr CIR was 5% in the late group patients (83% of which had received midostaurin) and 29% in the early group.

It is worth mentioning that *FLT3*-TKD mutations could not be extensively studied since in the CETLAM group they were only available from 2015 and were virtually absent in the early cohort (n=2). Still, in patients from the late cohort with *NPM1*mut/*FLT3*-TKD (n=18), we observed a 2-yr OS of 72±10%.

Finally, we evaluated the outcome of patients with *FLT3*mut and a *NPM1wt* (n=75). Again, clinical characteristics and response rates were balanced between early and late cohorts, and 69% of late cohort patients (29/42) received midostaurin. Although the cohort size was limited, we did not observe differences in midostaurin naïve vs. exposed patients regarding EFS (10 months in both groups) or OS (2-yr OS 48±7% vs. 51±10% p=0.9), and the HR for OS of midostaurin in this subgroup was 0.95 (95% CI 0.51-1.79 p=0.9).

6. OVERALL SUMMARY OF THE DISCUSSION

AML is the most frequent acute leukemia in adults with varying prognoses according to multiple factors, with a central role in genetics. The advances in NGS and other genomic analyses have disaggregated the disease into multiple subcategories with different responses and prognoses, while the advent of target therapy from clinical trials to patient bedside is rapidly changing the standards of this disease.

Since the late 20th century, multiple collaborative efforts were directed to categorize the disease into different risk groups and progressively incorporated molecular markers. The most recent risk classifications developed by a highly experienced panel of experts include a broader number of single-gene mutations as prognostic markers. However, the interaction between them is still difficult to assess in most cases due to its unknown impact, and therefore they are mostly not considered in clinical decisions.

In the first article of this thesis, we analyzed the impact on treatment response, survival and MRD eradication of the combination of the most frequently mutated genes in adult AML: *NPM1*, *FLT3* and *DNMT3A*. In the second article, we validated the impact of incorporating targeted therapy on *FLT3*mut in a homogeneous cohort of intensively treated patients.

For the first part of this thesis we analyzed 164 fit AML-*NPM1*mut patients treated from 2003 to 2017 and assessed their *DNMT3A* mutational status. It is noteworthy that when this study was performed, NGS was not routinely performed on AML patients and the genetic studies that were included aside from standard karyotype were the ones established by the ELN-10 classification (core-binding factor rearrangements, *MLL* partial tandem duplications, and mutations of *NPM1*, *FLT3*-ITD or TKD and biallelic *CEBPA*). Thus, in our study, we performed a directed PCR and Sanger sequencing to detect *DNMT3A* mutations and found them in almost half of the patients. Overall, *DNMT3A*mut did not modify the survival of *NPM1*mut patients. Several studies have tried to establish the prognostic impact of *DNMT3A*mut with contradictory results, possibly due to differences in the biological characteristics of the patients included (age, cytogenetics, availability of molecular studies) or the treatment protocols.(92, 103, 176, 177)

Our goal in this study was not to elucidate the prognostic impact of single-gene *DNMT3A*mutation on AML but to study how the combination of 3 mutations affected

patients. For this purpose, we first validated the impact of *FLT3*-ITD allelic ratio, an essential determination in our clinical practice for *NPM1*mut patients since it guides one of the major decisions in AML: to perform alloHCT in CR1 or not. Our group had previously described the prognostic value of the *FLT3*-ITD ratio in 2012 in work led by Dr. Pratcorona that included 303 patients treated from 1994 in earlier CETLAM protocols (AML-94, AML-99, AML-03).(80) Based on these results, since 2012, we have been using the allelic ratio to allocate patients to risk categories with the corresponding clinical consequences. In 2022 Dr. Bataller from our group published our results with this approach.(178) However, we needed to validate these findings in our study before incorporating *DNMT3A* mutations. In accordance with the previous publications, in this study, *NPM1*mut/*FLT3*low patients showed similar outcomes to *NPM1*mut/*FLT3*wt cases with a 5-yr OS of around 65% and CIR of around 15%. In comparison, *NPM1*mut/*FLT3*high patients presented a poorer outcome with OS and CIR at 5-yr of around 40%.

The interesting findings came when we incorporated *DNMT3A*mut in the equation. Although the allelic ratio of *FLT3*-ITD still maintained its discriminatory value, the prognosis of the patients differed whether they presented with the triple mutation (*DNMT3A/FLT3/NPM1*) or with only *FLT3*mut/*NPM1*mut. This difference was most relevant in the *FLT3*high subgroup, where their outcome dropped in the presence of *DNMT3A*mut to a 5-yr OS 28% and a 5-yr CIR of almost 50%. On the contrary, in the absence of *DNMT3*Amut the deleterious effect of FLT3high ratio was mitigated and their 5-yr OS increased to 47%.

On the other molecular subsets, the impact of DNMT3Amut was less notorious. Thus, *NPM1*mut/*FLT3*low had similar outcomes to patients with *NPM1*mut/*FLT3*wt regardless of their *DNMT3A*mut status. However, because an effect of *DNMT3A*mut on *NPM1* MRD clearance was demonstrated, we investigated the influence of an early intervention when molecular relapse was detected. Several publications in the last few years have analyzed the prognostic value of molecular MRD follow-up based on *NPM1* transcript levels. All of them support the prognostic impact of molecular MRD persistence with a higher incidence of relapse and shorter OS.(113, 179) The most extensive study evaluated the impact of MRD in peripheral blood after the second

chemotherapy cycle and reported that MRD persistence was the only independent prognostic factor for death in multivariate analysis.(124)

The ELN-17 guidelines were the valid recommendation during our study; they advised that a change in therapy should be considered in otherwise favorable patients with AML-*NPM1* and persistent MRD following a CR. Following the same reasoning, it was published by our group that an MRD ratio (*NPM1*mut/ABL1X100) of ≥0.05 (in bone marrow) after the CR1 was associated with significantly lower molLFS and that an early intervention resulted in a favorable outcome.(112)

The present study observed a trend towards worse molLFS in patients with *DNMT3A*mut, but without an impact on OS. When only patients in the favorable ELN-17 risk group were considered, we found that 27% met cytological or molecular relapse criteria. Of those, 70% underwent alloHCT in CR1 (in molecular relapse) or CR2. The effect of this strategy might counteract the negative effect on OS seen in the *DNMT3A*mut subgroup. This intervention might be the most crucial difference between our protocol and those included in the study by Papaemmanuil et al. (5), which considered alloHCT only in patients with a high cytogenetic risk. In contrast, intermediate-risk patients underwent alloHCT only when a sibling donor was available.

Overall, with this study, we concluded that patients with AML and the combination *NPM1*mut/*DNMT3A*mut/*FLT3*low could still be classified as favorable risk. Closer MRD follow-up is recommended to detect a molecular relapse and proceed to a therapeutic intervention. On the contrary, patients with *NPM1*mut/*DNMT3A*mut/*FLT3*high were identified as a very high-risk group.

The second part of this thesis arises from an observation that we came across in 2022, while performing other molecular analyses from the CETLAM AML-12 cohort. We observed that the survival of *FLT3*mut patients was persistently different in the late years, and the survival of *FLT3*-ITD appeared impressively improved from our previous publication. Considering that the only significant modification introduced in the protocol was the addition of the FLT3 inhibitor midostaurin from 2016, we thought it was essential to explore the changes in the prognosis of *FLT3*mut patients before and after the advent of midostaurin and to see if it validated (or not) the value of the *FLT3*-ITD allelic ratio.

For this instance, we studied 227 de novo *FLT3* mutated AML patients fit for intensive therapy homogeneously treated within the CETLAM AML-12 phase II trial. In the trial, post-remission therapy was based on the patient's genetic risk at diagnosis and MRD evolution. The intention to treat was alloHCT in case of intermediate and high-risk genetic categories or persistence of MRD in the favorable AML group.

Patients were divided into two groups, those treated between 2012 and 2015 and patients treated between 2016 and 2020 when midostaurin was available. We observed a remarkable improvement in survival during the last period due to a significant decrease in relapse incidence Overall, our study confirms the benefit of midostaurin in patients with AML and *FLT3*mut eligible for intensive chemotherapy, a subgroup that includes 30% of adults with this disease and is associated with adverse outcomes.

In our experience, the prognostic improvement was predominantly observed in patients with *NPM1* co-mutation and no benefit was found in the absence of *NPM1*mut. Patients with *NPM1*mut/*FLT3*low had an extremely favorable outcome with 81% survival at two years, and 64% were never transplanted. Even patients with *FLT3*high had a 57% survival at two years in the presence of an *NPM1* co-mutation.

A few other studies have analyzed the impact of midostaurin in *FLT3*-mutated AML but had several differences from our report. The seminal RATIFY trial included patients up to 59 years; ours extends this up to 70 years.(168) Another study evaluated the results of standard chemotherapy (intensive or non-intensive) with the addition of other FLT3 inhibitors, such as sorafenib or quizartinib.(180) Finally, a recent study from the German-Austrian AML study group compares the results in *FLT3*-ITD mutated AML with historical controls from five trials since 1990 and with the standard arm of the RATIFY trial.(181) In contrast, our study has the advantage of analyzing patients from the last decade, homogeneously treated by the same group of investigators, and the only variable that may justify the significant improvement was the introduction of midostaurin. Of note, we analyzed patients without *FLT3* mutations treated between 2012 and 2020, and no improvement in relapse or survival was observed (data not shown).

From the beginning of 2012, the CETLAM group did not consider *NPM1*mut*/FLT3* favorable (wt or low) patients for alloHCT in CR1. This premise was incorporated in

the ELN-17 recommendations but removed in the latest ELN-22 classification, where *NPM1*mut/*FLT3*low patients are considered intermediate-risk and recommended for alloHCT in first remission. This study highlights the positive change in the prognosis of *NPM1*mut/*FLT3*mut patients due to the association of intensive treatment and targeted therapy with midostaurin, and it validates the role of the *FLT3* ratio in identifying a highly favorable subgroup of patients.

In the AML-12 trial, in all intermediate and adverse patients the intention was to proceed to alloHCT unless major complications arose during chemotherapy, and in the favorable genetic category, only patients with a cytological relapse or those in remission but with persistent or reappearing MRD were considered for alloHCT. All these interventional strategies did not allow a fair assessment of the impact of alloHCT in the multivariable analysis. When we made an exploratory assessment of the impact of transplantation in the favorable ELN-17 subgroup (*NPM1*mut with *FLT3*low or TKD) and performed a Cox regression with transplantation as a time-dependent covariate, a worse survival in transplant recipients was observed that we consider was attributable to the worse characteristics of transplanted cases (MRD positive or in relapse).

Finally, TKD mutations were only available in the late cohort, and therefore their outcome could not be comprehensively contrasted. Additional division of ITD/TKD subsets as well as the impact of other co-mutations resulted in too few patients in each subgroup to provide statistical significance in the current analysis and should be explored in larger studies. On the other hand, additional progress is needed in the *NPM1*wt/*FLT3*-ITD group, since midostaurin in our hands did not improve their outcome.

In conclusion, the advances in understanding the complex mutational landscape of AML and its clonal evolution will undoubtedly translate into an improvement in MRD monitoring, treatment of AML, relapse-prevention and survival improvement.

7. CONCLUSIONS

1. *DNMT3A* mutations do not modify the favorable prognosis of AML patients with mutated *NPM1* both in the absence of *FLT3*-ITD or with the co-occurrence of *FLT3*-ITD low ratio, but the triple association of *NPM1*mut/*DNMT3A*mut and *FLT3*-ITD high ratio represent a particularly adverse group.

2. Closer MRD monitoring is recommended in the favorable-risk subsets of AML-*NPM1*mut that associate *DNMT3A* mutations in order to detect molecular relapses and proceed to early-therapeutic interventions.

3. The incorporation of midostaurin to intensive therapy in 2017 significantly improved the outcome of AML fit patients with *FLT3* mutations compared to early-treated patients from the same protocol.

4. The benefit of midostaurin is most prominent in the subgroup of *NPM1*mut/*FLT3*low patients, and our results endorse delaying alloHCT for patients in CR2 or molecular relapse.

5. The adverse prognosis of the known adverse group, *NPM1*mut/*FLT3*-ITD high ratio, is highly mitigated with the strategy of intensive therapy plus midostaurin and alloHCT in first CR.

8. FUTURE LINES OF INVESTIGATION

Major advances have developed in the diagnosis and treatment of AML during the last ten years that have led to the elaboration of new disease and risk classifications and the initiation of multiple clinical trials for fit and unfit patients.

With the incorporation of more molecular markers to the latest classifications of AML new questions arise. Thus, at the translational level our next projects will focus on:

1. Validation of the ELN-22 classification in the CETLAM cohort of intensively treated patients up to 70 years.
2. Report of NGS results in fit AML patients to correlate co-mutational patterns with treatment response.
3. Single-cell mutational analysis of selected patients (possibly focusing in ASXL1 and/or RUNX1 mutations) at diagnosis, complete remission and relapse to understand clonal evolution.
4. Validation of the NGS MRD strategy to achieve a better clonal follow-up.

Regarding therapy, we are currently in an early phase of developing our own trial, called the VENEFIT trial, a phase 3 randomized, open-label, multicentre study testing initial therapy with aza-venetoclax vs. intensive therapy in fit patients with AML or MDS/AML potentially eligible for allotransplant. The aim is to explore whether aza-ven could achieve at least the same CR rates than intensive chemotherapy, with potentially less toxicities and in a mostly outpatient setting which will overall improve quality of life of AML patients during the remission-induction phase of treatment.

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10. ANNEXES

10.1. Communication to international congress

Improved outcome of patients with acute myeloid leukemia harboring FLT3 mutation in the era of targeted therapy. Guadalupe Oñate, Marta Pratcorona, Ana Garrido, Alicia Artigas, Alex Bataller, Mar Tormo, Montserrat Arnan, Susana Vives, Rosa Coll, Olga Salamero, Ferran Vall-Llovera, Antònia Sampol, Antoni Garcia, Marta Cervera, Sara Garcia Avila, Joan Bargay, Xavier Ortín, Jordi Esteve and Jorge Sierra.

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P565 IMPROVED OUTCOME OF PATIENS WITH ACUTE MYELOID LEUKEMIA HARBORING FLT3 MUTATION IN THE ERA OF TARGETED THERAPY

Topic: 04. Acute myeloid leukemia - Clinical

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Background: In recent years, the treatment of AML is rapidly evolving due to the advances in targeted therapy, risk-adapted protocols and MRD-guided decisions. The prognosis of patients harbouring *FLT3*-ITD differs according to its allelic ratio and the presence of *NPM1* co-mutation (*NPM1*mut). Nonetheless, *FLT3*-ITD prognostic impact and allogenic hematopoietic cell transplantation (alloHCT) indication in this setting might be redefined by the widespread use of *FLT3* inhibitors. Since 2012, chemotherapy eligible patients are treated following a risk-adapted and MRD-guided protocol in the CETLAM group (AML-12). Since 2016, targeted therapy with midostaurin was progressively incorporated in the protocol for *FLT3*-mutated (*FLT3*mut) patients.

Aims: To analyse the outcome of *FLT3*mut AML treated with a risk-adapted protocol in two different time periods, mainly differentiated by the advent of midostaurin.

Methods: All adult patients with *de* novo AML and available *FLT3* status treated with the intensive protocol AML-12 (clinicaltrials.gov #NCT04687098) in the Spanish CETLAM centres from 2012 to 2020 were retrospectively analysed. Molecular testing of *NPM1*, and *FLT3*-ITD and its allelic ratio were studied as previously described. Patients were divided into two study cohorts: the early (2012-2015) and the late cohort (2016-2020). All patients received the same chemotherapy protocol except for the addition of midostaurin since 2016 in most *FLT3*mut patients. Complete remission (CR), overall survival (OS), event-free survival (EFS) and risk of relapse (RR) followed ELN criteria. OS and EFS were studied with the Kaplan-Meier method and log-rank test, cumulative incidence and Grey test were used to estimate relapse risk (CIR) and non-relapse mortality (NRM).

Results: A total of 906 patients were selected, 390 from the early and 516 from the late cohort. Median follow-up was 38 months; *FLT3*mut was present in 227 cases (25%) and constitute the focus of this study. There were not differences on main variables between cohorts (age, cytogenetic risk, ELN category, frequency of *NPM1* mutation or *FLT3*-ITD allelic ratio). TKD mutations were only available since 2015. Midostaurin was administered to 62% of *FLT3*mut patients from the late cohort: 63 with ITDs (20 low and 43 high ratio) and 20 TKDs. CR rates were similar between cohorts. AlloHCT in first CR (CR1) was performed in 60% (early) and 67% (late) patients (p=ns). A higher RR was observed in the early group in comparison to the late cohort (2-year RR 42±11% vs 28±10%; p=0.014), without differences in NRM (Figure 1B), translating into an improved EFS (median EFS 9.4 vs. 26 months, p=0.014) and OS in the late cohort (median OS: 15 months vs. non-reached, p=ns, Figure 1A). Among *NPM1*mut AML patients, *FLT3*-ITD and ELN 2017 categories retained its prognostic value in the early cohort, with a worse

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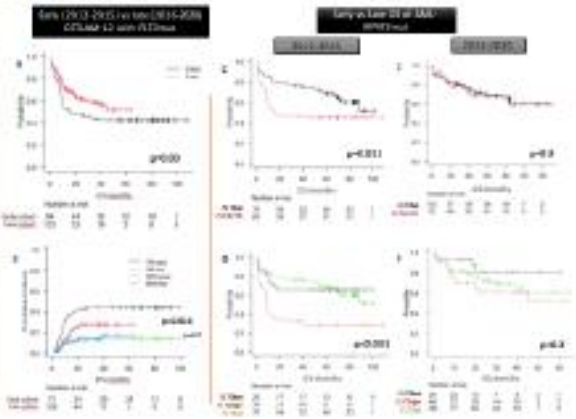
OS, EFS, and higher RR among *FLT3*-ITD vs wt patients (HR for OS 1.9 95% CI 1.15-3.2, p=.011; figure 1C) and *FLT3*mut ELN-intermediate vs favourable patients (OS p<0.001; figure 1D). On the contrary, in the late cohort, *FLT3*-ITD and *FLT3* high allelic ratio lost their adverse prognostic impact, with comparable RR, EFS, and OS regardless of *FLT3*-ITD status (Figure 1E and F).

Image:



Summary/Conclusion: This real-life study demonstrated an improved outcome among *FLT3*mut AML patients treated in the most recent period, probably reflecting the beneficial effect of midostaurin on relapse prevention in this context. Future analyses should revise the role of alloHCT in CR1 for non-favourable *FLT3*mut AML patients treated with *FLT3* inhibitors who achieve MRD eradication.

10.2. Financial support

The research presented in this thesis has been carried out in the Group of Hematology and Transplantation from the Institut d'Investigació Biomèdica Sant Pau (IIB-SANT PAU) and the Hematology Department of the Hospital de la Santa Creu I Sant Pau in Barcelona. In addition, all studies have been conducted with the collaboration of the Spanish CETLAM Group (Grupo Cooperativo de Estudio y Tratamiento de las Leucemias Agudas y Mielodisplasias).

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This work was also supported by grants "La Marató de TV3" Foundation 2019 41-30, AGAUR 2017 SGR 1395 and 2021 SGR 01139 from the Catalan Government and a grant from "Obra Social La Caixa" Barcelona Spain. PERIS SLT002/16/0043 from the Catalan Government and FIS PI17/01246, PI20/01621 and RD16/0011/0028 from the Instituto de Salud Carlos III, Ministerio de Economía y Competitividad, Spain.

10.3. Questions and answers from reviewers from first article

Reviewer #1:

Onate et al. submit a very intriguing analysis of survival outcomes in *NPM1* mutated AML subgrouped by *FLT3* and *DNMT3A* mutations. This is an important problem to address because Papaemmanuil analysis of German studies suggested a strikingly poor outcome in patients with the triple mutation, and multiple papers have suggested that persistence (MRD) of *DNMT3A* mutations during remission does not impact survival outcomes. Therefore, this is timely and clinically interesting.

The authors identify 164 patients with *NPM1* mutations on their studies. They find that *FLT3*-ITD allelic ratio, but not the presence of *DNMT3A* mutations correlated with outcomes. However, they find that cases with *DNMT3A* mutations tended to have higher *NPM1* transcript burden (MRD) after induction and slower clearance of these transcripts. This does not seem to impact overall survival, but these patients were treated with an early therapeutic intervention when *NPM1* transcripts began to rise.
Concerns:

1. It may be helpful to provide some survival figures that situate the overall survival of these subsets against the broader patient populations in the studies. This analysis could be supplemental and need not be extensive. Presumably, the survival looks a lot like intermediate risk AML. Overlaying other patients on some of these graphs will give a sense of effect size and relevance.

Response

As suggested, we performed a new Kaplan Meier analysis to contrast the OS and LFS of all CETLAM-12 patients (non-APL AML <70yr treated intensively) and our sub-cohort (AML-*NPM1*) divided according to their DNMT3A mutational status. This figure will be added to supplemental material as Supplemental Figure 2 and is referenced in the main text.

Of note, Next Generation Sequencing was not routinely established until the end of 2017, and consequently patients in the intermediate-risk group, but with mutations in *RUNX1, TP53* or *ASXL1* were mainly not identified and remained included in the intermediate-risk group. Thus, we cannot provide a reliable classification based on

ELN 2017 criteria, and in this analysis, non-*NPM1* AML-12 patients are stratified according to the risk classification (favorable, intermediate or adverse) established in the protocol available at clinicaltrials.gov. In summary, it follows ELN 2017 recommendations except for the identification of adverse risk mutations (i.e. RUNX1, ASXL1 and TP53). The presence of these patients, that from the last ELN classification are included in the high-risk group, added to the absence of *NPM1*^{mut}/*FLT3*-ITD high patients that are included in the study, may be an explanation why patients in the adverse-risk group have a worse outcome than would be expected.

2. The p-values presented in the survival curves appear larger than one might expect just "eyeballing" the data. The statistical tests used are not clear. The methods just mention Mann-Whitney U tests. Log-rank tests should have more power to detect time-to-event data. Perhaps the authors could clarify the tests used.

Response: To clarify the statistical analyses performed, we added a broader description in the methods section (page 5, Methods section: Statistical analysis).

3. In these trials, early intervention was used following *NPM1* MRD rise. The discussion should more completely discuss differences in outcomes between this study and other studies, and how this intervention might have affected these outcomes.

Response: This is a very interesting point, which we emphasized in the discussion adding the following paragraph:

"In the last few years, several publications analyzing the prognostic value of MRD follow-up based on NPM1 transcript levels have been published. Although there is not a consensus about the cut-off level and the evaluation time points, all of them consider that MRD positivity persistence has a prognostic impact with a higher incidence of relapse and a shorter OS.(112, 113, 179, 182, 183) The largest study performed(124) evaluates the impact of MRD positivity in peripheral blood after the second chemotherapy cycle, and they find the same impact in prognosis as previously reported, but they also report that MRD persistence is the only independent prognostic factor for death in multivariate analysis. The ELN recommendations (127) also consider that in

the AML-NPM1, the failure to achieve an MRD negative CR, or rising MRD levels are associated with disease relapse and consequently advise that a change in therapy should be considered. Following the same reasoning, it was recently published by our group that an MRD ratio (NPM1mut/ABL1X100) ≥ 0,05 (in bone marrow) after the first consolidation cycle was associated with a significantly lower molLFS, and that an early intervention resulted in a favorable outcome (112) Consequently, MRD level can be considered a good strategy to be used as a guidance of post-remission therapy."

Reviewer #2:

This paper makes some important observations about the interaction between *DNMT3A*, *FLT3* and *NPM1* mutations. This interaction was previously demonstrated by Papaemanuil et al in a very large cohort, amongst others. This manuscript provides valuable confirmation of these findings but differs in one important way namely that *DNMT3A* mut / *FLT3* ITD low patients show no difference in overall survival time compared to *DNMT3A* WT patients. If I understand correctly, given that the molecular leukemia free survival rates show a trend to difference the authors suggest that the discrepancy between the studies could be due to MRD-guided intervention.

1. The discussion is slightly difficult to follow and would benefit from some revision and in particular to highlight the similarities and differences with the AMLSG study and others, and the suggested reasons underlying these discrepancies.

Response: Following the recommendations of the reviewers we rewrote all the discussion and added a paragraph trying to explain the differences with the AMLSG publications:

"Interestingly, in the present study a trend towards a worse molLFS was observed in patients with DNMT3A^{mut}, but without an impact in OS. When only patients of the ELN-2017 favorable risk group were considered, we found that 27% of patients met either cytological or molecular relapse criteria. Of those, 70% received an alloHSCT in CR1 (but in molecular relapse) or second CR, and the effect of this strategy might counteract the negative effect in OS of the DNMT3A mutated subgroup. This intervention might be

the most important difference with the treatment protocols of the patients included in the Papaemmanuil and colleagues study, which considered an alloHSCT only in high cytogenetic risk patients, while intermediate risk patients underwent to an alloHSCT only when a sibling donor was available"

2. There is a concern about the definition of MRD negativity used in this study. According to the European leukemia network, MRD negativity is defined as no amplification with a cycle threshold of <40 in two of three replicates. In this work the authors have used a definition of *NPM1* copiesx104 /ABL1 copies of <0.01. The authors should reanalyze their data using the correct definition of negativity. Please could the authors check whether this affects their results in any way? Following on from this comment, I wonder if instead of figure 4B (which is a bit confusing) the authors might want to show how many patients are MRD negative in each group at the post induction and post consolidation 1 time point? Or perhaps this could form a useful supplemental figure?

Response: Following reviewer 2 interesting remarks, we reanalyzed our MRD data. We classified patients after each treatment course according to their MRD status (positive vs. negative), applying the ELN definition as suggested (no amplification with a cycle threshold of <40 in two of three replicates). We compared MRD status in both *DNMT3A* subsets (mutated and wild type) through a Chi-square test. Interestingly, no differences were found after one cycle of treatment (induction), but apparent differences were observed after each consolidation cycle.

When MRD log10 reduction was analyzed with the MRD positive/negative results, it was shown that *DNMT3A*wt patients not only were more frequently MRD negative, but they also achieved more profound responses. With these results, we formed the definitive 4B figure in replacement of the previous one. We also modified in the same terms supplemental figure 5 to show MRD response following the third consolidation. Accordingly, this new analysis has been included in the main text.

3. Finally, it would be of interest to see the rates of MRD clearance specifically in the triple mutated group (either or both of when defined as any *FLT3* ITD and when defined as ITD >0.5) and this could perhaps form a useful supplementary

figure?

Response: In our cohort, 28 patients presented with AML-*NPM1*, *DNMT3A*mut and *FLT3*-ITD (11 low ratio, 16 high ratio and 1 unknown ratio). Although our data was limited, all the triple mutated patient with MRD information were MRD positive following induction (13/13), consolidation-1 (13/13) and consolidation-2 (9/9). Only one patient achieved MRD negativity following third consolidation as summarized in the following table.

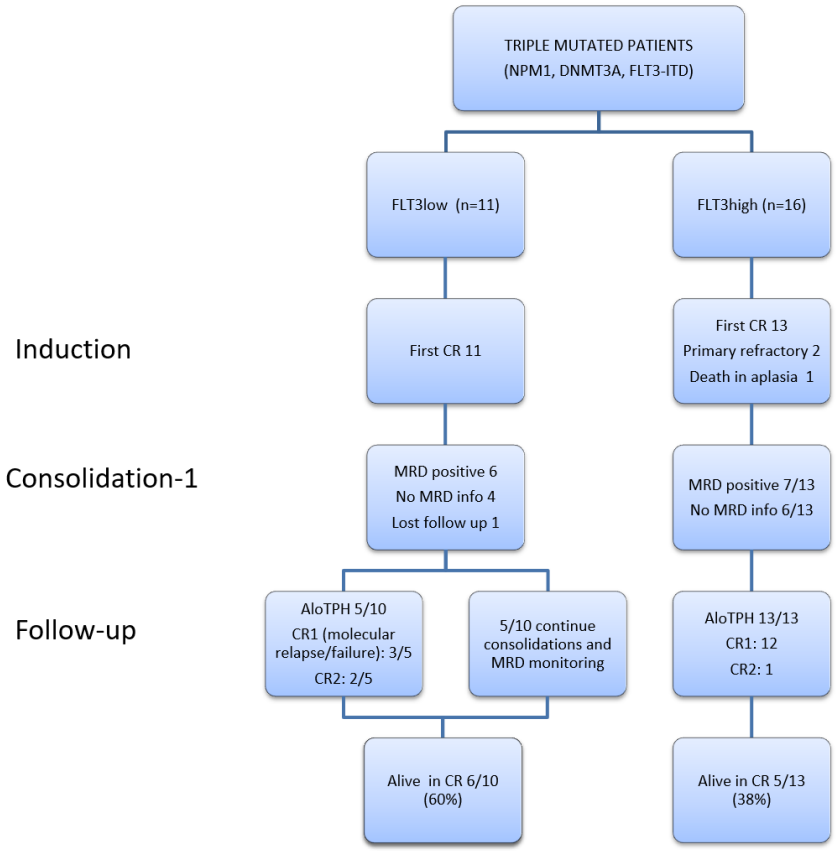
	***DNMT3A*^{mut}*FLT3*^{low}**	***DNMT3A*^{mut}*FLT3*^{high}**
MRD+ post-induction (n)*	6/6	7/7
MRD+ post-C1 (n)*	6/6	7/7
MRD+ post-C2 (n)*	5/5	4/4
MRD+ post-C3 (n)*	4/5 (1/5 MRD negative)	0$^{\#}$

* (number of patients with available MRD information)

All high ratio patients either received an allogeneic transplant following C1 or C2 or were refractory/relapsed/death in induction and were excluded from protocol.

MRD+: positive measurable residual disease; CR: complete remission, C1: consolidation-1; C2-consolidation-2; C3- consolidation-2;

We summarized this information in the main text, and we supply here a diagram that summarizes the clinical course of these patients.



Finally, the re-analysis of our MRD data led us to review the molLFS section, and we decided to add a supplemental figure 6 detailing the molLFS of the whole cohort to improve clarity that is also referenced in the main text.

Reviewer #3:

The authors studied 164 younger adults with NPM1-mutant AML who received intensive therapy on consecutive protocols in the Spanish cooperative group following ELN guidelines. The known prognostic value of FLT3 allelic ratio was confirmed. The impact of DNMT3A mutations on MRD clearance and prognosis within FLT3 subsets was examined. Comments:

1. The prognostic value of *DNMT3A* mutations in AML has been extensively studied (PMID: 21670448, 2228988, 22490330, 22291079, 23632886,

23962568) often in much larger cohorts than the one examined here. Although there is limited consensus across these studies (including ones that focus on NPM1 and FLT3 co-mutation status), I do not think the current study is adequately powered to provide definitive answers. At the least, the discussion should clearly state areas of agreement and disagreement with the existing literature.

Response: We completely agree with this comment, and we add a clarifying paragraph to our discussion:

Page 9, first paragraph of discussion:

"Several studies have been published trying to elucidate the prognostic impact of DNMT3A mutations, but many of them have contradictory results. It may be due to the differences among the biological characteristics of the patients included (age, cytogenetics, availability of molecular studies, etc.), the treatment protocols, or other confusing factors. Of note, even those studies comparing the impact of DNMT3A considering NPM1 and FLT3 mutational status show contradictory results.(92, 103, 177) The aim of this study was not to analyze the impact of DNMT3A in AML outcome, but only to analyze its effect in the particular subset of patients with NPM1^{mut} and FLT3-ITD, after the publication of a large study showing that DNMT3A mutations have a deleterious effect on outcome when co-occurring in this subgroup.(5) Our group described the effect of FLT3-ITD ratio in 2012, and it was incorporated in the new treatment protocol. Consequently, patients with NPM1^{mut} and FLT3^{low} did not undergo to an alloHSCT in CR1. Therefore we had a long follow up in this group of patients treated following the ELN-2017 recommendations, to analyze the possible effect of DNMT3A mutations."

2. The authors claim that DNMT3A mutations have an adverse impact in the *FLT3*-ITD (high) group (2nd key point, p. 7 of results), but there is no direct comparison of *DNMT3A* wt vs. mutant in the *FLT3*-ITD (high) group in Figures 2 or 3. Furthermore, the authors state in the Discussion (p 9) that this difference does not reach statistical significance. As a practical consideration, this finding

(if statistically robust) would not be actionable since these patients are already in a high risk group.

Response: Following reviewer 3 remarks we changed the key points, results and discussion. We also added the requested figure as supplemental figure 4.

We agree with the reviewer's comment on the current non-actionable condition of potential negative impact of DNMT3A mutation in high-ratio FLT3-ITD AML patients. Nonetheless, the identification of a very poor risk subset, with a highest relapse risk, could always constitute a target population for innovative approaches with experimental agents or strategies. Therefore, considering the lack of prognostic significance of DNMT3A mutation on LFS and OS in this study, we replaced the previous 2nd key point with a statement highlighting the relevant effect of DNMT3A mutation of MRD clearance.

3. The MRD analysis is perhaps the most interesting and novel part of the manuscript. Here, *DNMT3A* mutations appear to be associated with delayed MRD clearance in the FLT3/NPM1 favorable groups. As the authors point out, this did not impact LFS/OS implying that salvage therapy/alloHCT can overcome this unfavorable feature. It might make for a more compelling presentation of the results if the authors led with the MRD analysis and then showed the LFS/OS data stratified by DNMT3A status in Figures 2 and 3 as they do in Figure 1.

Response: In agreement with the reviewer, the effect of *DNMT3A* mutation is mostly observed in the delayed MRD clearance (Fig. 4). The lack of an overt clinical impact in terms of LFS or OS might be the result of the treatment protocol design, aimed to implement a preemptive intervention in patients experiencing a molecular failure.
We have now emphasized this deleterious effect of *DNMT3A* mutation on *NPM1*-MRD kinetics in several sections of the manuscript, including the Abstract, Results and the Discussion section, and it has now been included among the key points.

In addition, we performed the analyses suggested by the reviewer, with stratification of main outcomes by *DNMT3A* mutational status. The resulting figures are shown and have been included in the supplementary material (supplemental figure 4, previously commented). One of the main focuses of the study was to analyze the cohort of *NPM1*^{mut}/*FLT3*^{low}, in order to confirm its comparable outcome to *NPM1*^{mut}/*FLT3*^{wt} and identify/discard a poorer prognosis subgroup defined by the presence of DNMT3A co-mutation. This was the reason underlying the comparative analysis performed in Figures 2 and 3, focusing on these *NPM1*/*FLT3*-ITD subgroups.

10.4. Questions and answers from reviewers from second article

Reviewer #1:

Onate et al summarized the outcome of 227 patients with newly diagnosed FLT3-mutated AML from 2012 to 2020 using the AML-12 prospective trial data. From the early time period 2012-2015 to the late time period 2016-2020, the 2-year incidence of relapse has improved from 42% to 29%; the 2-year survival, 47% to 61%. The improved outcome was observed in FLT3-mutated and NPM1-mutated AML with the mitigation of higher allelic burden of FLT3 mutations. The outcome did not improve in patients with FLT3-mutated and NPM1 wild type AML. This article confirmed the benefit of FLT3 inhibitors in patients with FLT3-mutated NPM1-mutated AML using an independent dataset.

Major:

It is interesting to see no survival benefit of midostaurin in patients with *FLT3*-mutated *NPM1* wild type AML.

a. Given the high-risk ELN AML, 72% patients proceeded to allogeneic stem cell transplant. How many did patient receive post-SCT midostaurin maintenance therapy?

Response: None of the *NPM1*wt patients received post-SCT midostaurin maintenance. Only one *NPM1wt*/*FLT3*mut (high ratio) patient received midostaurin maintenance and it was due to complications during consolidation chemotherapy that contraindicated SCT. The description of the patients who received maintenance is described in lines 183-188 of the main text, and briefly exposed in figure 1.

b. It might be possible that the midostaurin exposure is very limited in the pre-SCT period which may lead to non-significant difference in outcomes. What is the median time of midostaurin exposure? I recommend writing a paragraph in Discussion.

Response: Midostaurin exposure is something that we did not look at. Thanks to reviewer-1 remark, we revisited all our patient's data on this aspect in the cooperative database. We were able to gather information from all patients in induction-1, induction-2 and consolidation-1. We had no information regarding exposure in further cycles although most patients proceeded to transplant after consolidation-1 and because of that we consider that the data available are representative. In this regard, *NPM1*mut patients received a median of 25 days (4-28) of midostaurin, while *NPM1*wt patients a median of 18 days (8-28); this difference was not statistically significant and we incorporated this information in line 363 of the discussion as follows:

"On the other hand, additional progress is needed in the *NPM1*wild/*FLT3*-ITD group, since midostaurin in our hands did not improve their outcome. This particular finding should be weighed carefully due to the limited size number. We analyzed a possible impact of midostaurin exposure in the pretransplant setting in *NPM1* mutated vs wild type and found that the median number of days in the first group was 25 vs 18 in the second. This difference, however, was not statistically significant"

The analysis of the midostaurin exposure indicated by reviewer 1 was also helpful to detect a minor discordance in the data. Therefore, we corrected a misclassification of two patients (one *NPM1*wt and one *NPM1*mut) that were wrongly assigned as exposed to midostaurin. These patients were switched to the naïve group whereas all other patients were correctly allocated We repeated all survival analyses finding even more significant differences favoring midostaurin in the overall cohort and in the *NPM1*mut subgroup; of note, the outcome of *NPM1*wt patients remained the same regardless of midostaurin exposure. All the changes are highlighted in the main text for review, and figures 1, 3, 4, supplemental figure 8 and the graphical abstract were re-done.

2. In patients with *FLT3*-mutated *NPM1* wild type AML, how many patients received midostaurin?

Response: Line 265. Twenty-nine out of 42 late cohort patients received midostaurin (69%).

3. In Method, the authors stated allo-SCT was analyzed as a time-dependent variable. However, the Table 2 did not include allo-SCT. Given the known benefit of allo-SCT in FLT3-mutated AML, allo-SCT needs to be incorporated in the Cox prognostic models.

Response: Thank you for the comment, it is correct that we initially analyzed alloHCT as time-dependent variable as described in methods, but we did not finally include this covariate in the multivariable analysis since in our protocol transplantation was an evolutive risk-adapted strategy for adverse features cases. Specifically, in the CETLAM AML-12 trial the indication of alloHCT was defined according to risk at diagnosis and MRD evolution; in all intermediate and adverse patients the intention was to proceed to alloHCT unless major complications aroused during chemotherapy and in the favorable genetic category only patients in remission with persistent or reappearing MRD or those with cytological relapse were considered for alloHCT. All these interventional strategies did not allow, in our view, a fair assessment of the impact of alloHCT in the multivariable analysis.

In fact, when we made an exploratory assessment of the impact of transplantation in the favorable ELN-17 subgroup (*NPM1*mut with *FLT3*low or TKD) and performed a Cox regression with transplantation as time-dependent covariate, we observed a worse survival in transplant recipients (HR for OS (Allo) 4.71 95%CI 1.31-16.95 p=0.018) that we consider was attributable to the worse characteristics of transplanted cases (MRD positive or in relapse).

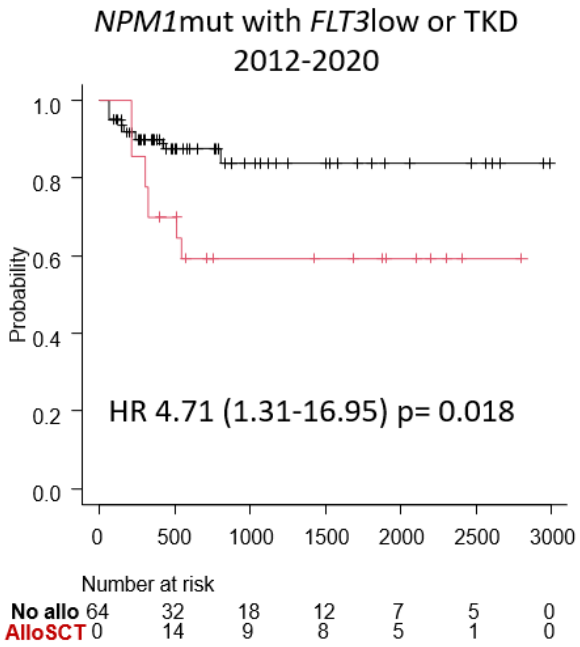


Figure: AlloHCT impact on patients with *NPM1*mut and *FLT3*-ITD low ratio analyzed with Cox proportional Hazard regression (HR) with time-dependent co-variate and represented with Mantel Byar graphs.

Similarly, in the *NPM1*mut/*FLT3* high subset, the impact of alloHCT was not assessable because the intention-to-treat in the protocol was alloHCT for all patients. In this category, among the 18 non-transplanted patients, 10 had died during induction and of the remaining 8 cases: 2 had refractory disease, 3 achieved CR1 but progressed and died before transplant, 2 were lost to follow up after CR1, and one had an autologous transplant per institution decision.

In summary, we believe that the real benefit of alloHCT should be evaluated in patient's subsets where transplantation or alternative treatments would be options based on randomization or donor/no donor availability (an unlikely scenario since nowadays almost all patients have a potential donor).

Finally, we summarized this information and included it in the discussion as follows (line 316):

"In the AML-12 trial the indication of alloHCT was defined according to risk at diagnosis and MRD evolution; in all intermediate and adverse patients the intention was to proceed to alloHCT unless major complications aroused during chemotherapy and in the favorable genetic category only patients in remission with persistent or reappearing

MRD or those with cytological relapse were considered for alloHCT. All these interventional strategies did not allow, in our view, a fair assessment of the impact of alloHCT in the multivariable analysis. In fact, when we made an exploratory assessment of the impact of transplantation in the favorable ELN-17 subgroup (*NPM1*mut with *FLT3*low or TKD) and performed a Cox regression with transplantation as time-dependent covariate, a worse survival in transplant recipients was observed (HR for OS (Allo) 4.71 95%CI 1.31-16.95 p=0.018) that we consider was attributable to the worse characteristics of transplanted cases (MRD positive or in relapse)."

4. I believe patients with NPM1 mutations were classified into favorable and intermediate ELN-17 prognostic categories. These two categories should be based on low or high the allelic ratio of FLT3-ITD. However, the number of patients in Figure 4B did not match the number in Table 1. Please clarify.

Response: Agreeing with reviewer 1e, *NPM1*mutations were classified into favorable and adverse according to *FLT3* ratio, however the ELN-favorable category included a few *FLT3*mut patients with CBF rearrangements which justify the number disparity between table and figure 4B. Following reviewer 1 recommendation, we modified Table 1 and specified each ELN subcategory to avoid confusion as summarized here:

Extract of Table 1:

ELN-17 prognostic categories			0.8
Favorable	30 (32%)	45 (34%)	
RUNX1-RUNX1T1	3	3	
CBFB-MYH11	1	1	
*NPM1*mut/*FLT3*low	25	25	
*NPM1*mut/*FLT3*-TKD	1	16	
Intermediate	38 (40%)	57 (43%)	
*NPM1*mut/*FLT3*high	34	47	
*NPM1*wt/*FLT3*low	3	5	
*NPM1*wt/*FLT3*-TKD	1	5	
Adverse	26 (28%)	31 (23%)	
*NPM1*wt/*FLT3*high	23	24	
Other*	3	7	

*Two cases in the early period presented with t(6;9) while the other had a complex karyotype (CK), in the late period 2 patients had CK, 1 a t(6;9), 2 patients harbored mutation of TP53 along with NPM1mut, and 2 cases a mutated RUNX1 without NPM1mut.

5. How did the authors measure MRD? Is the presence of MRD prognostic for relapse and survival?

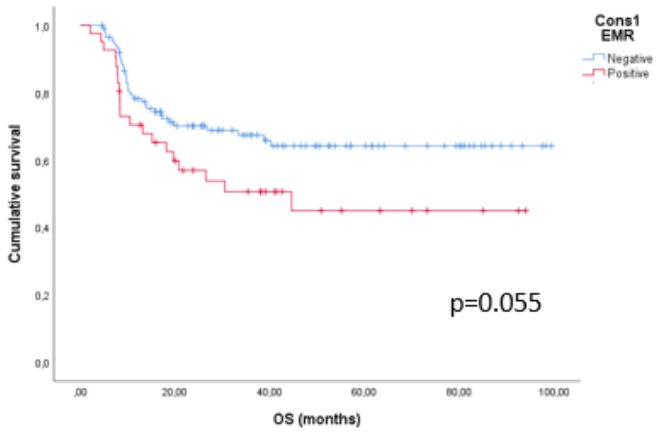
Response: MRD was measured in bone marrow samples after each treatment cycle. The methods used were either molecular monitoring of rearrangements *RUNX1::RUNX1T1* (threshold > 0.01% with respect to the diagnosis) and *CBF::MYH11* (threshold >10 copies with respect to 10^4 ABL copies), as well as *NPM1* transcripts as described by Gorello et al. Leukemia 2006 (threshold >200 copies with respect to 10^4 ABL copies) or multiparameter flow cytometry (threshold ≥0.1%).

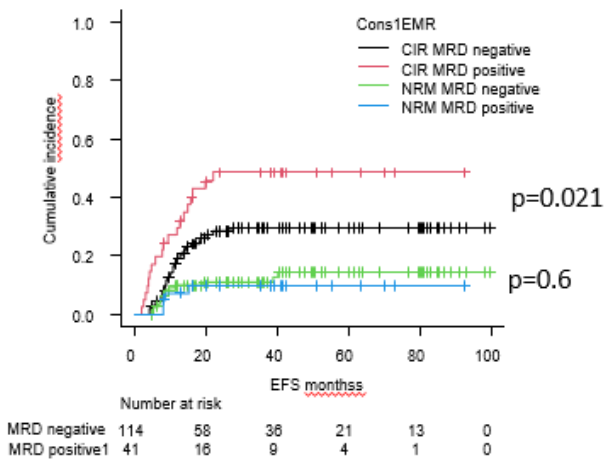
In order to respond reviewer-1 question, we evaluated our MRD results after consolidation-1 which corresponded to 2 courses of chemotherapy in >80% and in patients intended for alloHCT, and it was frequently equivalent to pre-transplant MRD. Data were available in 155 out of 172 (90%) patients in CR1.

We did not observe a significant association between between MRD negativity and time period (73% MRD negative in early and late cohort) or midostaurin exposure (74% in both naïve and exposed), neither in the whole cohort or in each *NPM1* subset.

In the overall cohort, post-C1 MRD persistence was associated with higher relapse-risk (2yr 48±15% vs 28±8% p=0.021) and a trend towards worse survival (2yr 57±8% vs 70±4% p=0.055).

We included the MRD description in Methods (lines 135-138), and incorporated this analysis in the Results sections (lines 197-202) while the figures were included in the supplemental material (supplemental figure 3)





6. In supplemental figure 2, the authors classified patients with TP53 mutations as favorable; a part of patients with RUNX1 as favorable. The RUNX1 could be co-occurred with favorable-risk AML. However, patients with TP53 mutations should be classified as adverse. If needed, it is better to repeat Cox regression models.

Response: Two patients harbored a *TP53* mutation in the presence of a *FLT3* favorable ELN subcategory (*NPM1*mut/*FLT3*low and *NPM1*mut/*FLT3*-TKD respectively), both with normal karyotype, one died during induction and the other was stratified as favorable in the protocol. We re-classified them as adverse, modified the supplemental figure 2 and repeated all multivariate Cox models (specified both in Table 2 and in the main text).

Minor:

1. In the abstract, the meaning of the description is not clear: Outcome was improved in the late period, with a 2-year relapse incidence and overall survival (OS) of 29% vs 42% (p=0.024) and 61% vs 47% (p=0.042). Which period is the early and late period? Readers would follow the natural time sequencing from early to late.
   Response: we incorporated group description to improve clarity: "Outcome was improved in the late period: 2-year relapse incidence decreased from 42% vs 29% in early vs late group (p=0.024) and 2-year overall survival (OS) improved from 47% vs 61% (p=0.042), respectively.
2. Define alloHCT in the first use. **Response:** In the main text it is now defined in line 76, in the abstract the acronym was removed.
3. Define CT in the first use. **Response**: It is now defined in line 97
4. How many patients had diploid (or -Y) in the early and late groups? **Response:** 67 seven patients (71%) in the early period and 90 patients (68%) in the late period presented with diploid karyotype.

5. Define Auto-TPH in Figure 1. **Response:** Thank you, AutoTPH is the Spanish acronym for autologous stem cell transplant, it is now corrected to autoSCT and we defined it in the figure legend.
6. In Figure 1, all the patients who received second induction apparently achieved CR/CRi in both early and late cohorts. Please clarify in text. **Response:** We modified figure 1 to improve clarity an added an explaining note below the figure. The post-induction response specified in the figure (CR, early death and refractory) was the final result after either induction-1 (in patients who only received one cycle) or induction-2 (in patients who achieved only partial response and received the second induction).
7. In the Early cohort of Figure 1, the number of favorable patients is 28. However, 18 patients received high-dose cytarabine; 8 patients proceeded to allo-SCT; 3 patients died of early relapse. The sum is 29. Please correct the mismatch. Response: We reviewed our data and found the mismatch: there were 2 (not 3) patients who presented death for early relapse. Of note, since ELN categories were slightly modified as mentioned above, we have modified the numbers in the figure accordingly.