UNIVERSITÀ
DEGLI STUDI
DI BRESCIA

Dipartimento di Scienze Cliniche e Sperimentali

Dottorato di Ricerca in “Intelligenza Artificiale in Medicina e Innovazione nella Ricerca Clinica e Metodologica”

Settore scientifico disciplinare: MED/15

Ciclo XXXVII

Feasibility Study on detection and monitoring of gene-mutations by digital (dPCR) NGS driven in patients with Acute Myeloid Leukemias (AMLs) and High-Risk Myelodysplastic Syndromes (HR-MDSs)- “dPCR-NGS Driven Study”

Nome del Supervisore:
Chiar.mo Prof. Domenico Russo

Nome del Dottorando:
Dr. Alessandro Leoni

Abstract in lingua italiana

La leucemia acuta mieloide (LAM) è una neoplasia ematologica caratterizzata da un'elevata eterogeneità biologica, clinica e prognostica. Nonostante con i programmi terapeutici attuali di induzione e consolidamento si possa ottenere la remissione completa (RC) nel 60-80% dei casi, la sopravvivenza a lungo termine è ancora limitata a causa dell'elevata frequenza di recidiva della malattia, che ne condiziona la prognosi e rende necessario, in gran parte dei casi, il ricorso al trapianto allogenico di cellule staminali ematopoietiche (T-allo-CSE).

Il rischio "genetico" determinato alla diagnosi attraverso l'impiego della citogenetica, della biologia molecolare e, più recentemente, dell'analisi mediante *Next Generation Sequencing* (NGS), unitamente alla determinazione e al monitoraggio della malattia residua misurabile (MRM) rappresentano, oggi, gli strumenti essenziali per la definizione della prognosi delle leucemie acute mieloidi e per il management più corretto e preciso del paziente affetto da LAM.

L'introduzione delle tecnologie di NGS ha permesso di osservare come il clone leucemico sia, in realtà, estremamente eterogeneo e caratterizzato da più sub-cloni, ognuno caratterizzato da mutazioni geniche con diverso significato prognostico, superando i limiti della citogenetica (metodica poso sensibile (10^{-2}), che risulta normale in circa il 50-60% dei casi) e anche quelli della biologia molecolare tramite RT-qPCR, una metodica altamente sensibile (fino a 10^{-5}) ma limitata alla rilevazione di pochi marcatori clonali di malattia (*FLT3*, *NPM1*, *IDH1*, *IDH2*).

L' NGS, tuttavia, pur avendo il vantaggio di permettere di definire il profilo genomico mutazionale delle LAM non è una metodica altamente sensibile nei pannelli commerciali attualmente in uso; quindi, è poco adatta a misurare l'MRM, visto che la sua sensibilità, fino a 10^{-2}, è ben al di sotto dell'analisi immunofenotipica (fino a 10^{-5}) la quale, tuttavia, può essere limitata da una significativa variabilità di espressione fenotipica degli antigeni malattia specifici, risultando poco affidabile per il monitoraggio della MRM.

Il presente progetto di tesi si propone di valutare la fattibilità e l'affidabilità di una metodologia innovativa per l'identificazione, la quantificazione ed il monitoraggio delle mutazioni associate alla LAM, basata sulla combinazione della digital Polymerase Chain Reaction (dPCR) guidata dall'NGS. Combinando l'analisi NGS, che permette di ottenere il profilo genico mutazionale alla diagnosi, con la dPCR, tecnica altamente sensibile ed accurata (fino a 10^{-5}/10^{-6}), si è cercato, da una parte, di definire il rischio genetico iniziale e,

dall'altra, di quantificare e monitorare nel tempo le mutazioni geniche correlate ai cloni leucemici individuati tramite NGS.

Il disegno dello studio è stato concepito affinché, al momento della diagnosi, venissero raccolti campioni biologici (sangue periferico e midollo osseo) da pazienti candidati a terapia intensiva, con l'obiettivo di effettuare l'analisi mutazionale mediante NGS (pannello mieloide, SOPHiA Genetics) e mediante dPCR, attraverso l'impiego di probes, detectare e quantificare le mutazioni NGS identificate per poterne successivamente monitorare l'andamento.

Le mutazioni "patologiche" o "potenzialmente patologiche" identificate mediante NGS sono state quindi successivamente monitorate tramite dPCR sia su campioni di sangue periferico che midollare raccolti a diversi *time points*: (i) dopo la terapia di induzione/consolidamento, (ii) al termine del trattamento e, (iii) nei pazienti sottoposti a trapianto di CSE, sia prima che dopo tale procedura. Per le mutazioni non note, in assenza di sonde commerciali disponibili per la dPCR, sono state disegnati specifici saggi personalizzati (*Custom TaqMan assays*). L'approccio *multiplex* della dPCR (QuantStudio™ Absolute Q™ Digital PCR System (Thermo Fisher Scientific, Waltham, MA, USA) ha consentito l'analisi simultanea di più mutazioni, con un significativo risparmio di tempo, costi ed una riduzione del margine di errore.

Inoltre, in caso di recidiva, era sempre prevista, in qualunque momento, la raccolta di campioni da sangue periferico e midollare per una nuova valutazione molecolare tramite NGS e dPCR, al fine di rilevare l'eventuale comparsa di nuovi cloni correlabili con l'insorgenza di resistenza della malattia alla terapia.

La fattibilità dello studio è stata definita come la possibilità di monitorare almeno il 70% dei pazienti mediante dPCR.

I risultati preliminari confermano la fattibilità dell'approccio dPCR NGS-driven per il monitoraggio dell'MRM. Infatti, l'86% dei pazienti (12 su 14) presentava almeno una mutazione identificata mediante NGS alla diagnosi, la quale è stata successivamente monitorata con successo tramite dPCR.

La maggior parte delle sonde (14/18; 78%) sono state customizzate, risultando paziente specifiche. Tuttavia, è importante sottolineare che l'iniziale alta percentuale di sonde *custom-designed*, potrebbe progressivamente ridursi in futuro, avendo a disposizione *probes* già disegnate per i pazienti analizzati.

Inoltre, tutte le mutazioni selezionate sono state rilevate sia nel sangue midollare che nel sangue periferico. Questo dato, in futuro, potrebbe suggerire la possibilità di monitorare la

MRM utilizzando esclusivamente campioni di sangue periferico. Lo studio ha quindi raggiunto il suo obiettivo principale, dimostrando la fattibilità dell'approccio combinato dPCR NGS-driven per il monitoraggio della MRM nella LAM.

Rispetto alla valutazione della MRM con metodiche tradizionali, i dati ottenuti evidenziano due possibili scenari: (i) monitoraggio della MRM in assenza di marker tradizionali, e (ii) monitoraggio della MRM in presenza di marker tradizionali. In quest'ultimo caso, l'approccio combinato basato su dPCR ed NGS ha permesso non solo di confermare i risultati ottenuti tramite l'analisi dei marker tradizionali, ma anche di anticipare la clearance molecolare e, in alcuni casi di rilevare precocemente o prevedere la recidiva di malattia.

In conclusione, l'approccio dPCR guidato da NGS si è dimostrato fattibile ed efficace per identificare, quantificare e monitorare le mutazioni associate alla malattia, rappresentando uno strumento avanzato per la valutazione della MRM e per una gestione più precisa e personalizzata dei pazienti affetti da LAM.

Abstract

Acute myeloid leukemia (AML) is a hematologic malignancy characterized by high biological, clinical, and prognostic heterogeneity. Although with current therapeutic programs of induction and consolidation, complete remission (CR) can be achieved in 60-80% of cases, long-term survival is still limited due to the high frequency of disease relapse, which affects its prognosis and necessitates the use of allogeneic hematopoietic stem cell transplantation (HSCT).

The "genetic" risk determined at diagnosis using cytogenetics, molecular biology, and, more recently, analysis by Next Generation Sequencing (NGS), together with the determination and monitoring of measurable residual disease (MRD) is now an essential tool for defining the prognosis of AML and for the more correct and accurate management of these patient.

The introduction of NGS technologies has made it possible to observe that the leukemic clone is, in fact, extremely heterogeneous and characterized by multiple sub-clones, each characterized by gene mutations with different prognostic significance, overcoming the limitations of cytogenetics (which is normal in about 50-60% of cases with mutations detectable by NGS) and also those of molecular biology by RT-qPCR, a highly sensitive method (up to 10^{-5}) but limited to the detection of a few clonal markers of disease (*FLT3*, *NPM1*, *IDH1*, *IDH2*). NGS, however, while having the advantage of allowing the mutational genomic profile of AML to be defined, is not a highly sensitive method in the commercial panels currently in use; therefore, it is poorly suited for measuring MRD, as its sensitivity, up to 10^{-2}, is well below immunophenotypic analysis (up to 10^{-5}) which, however, can be limited by significant variability, making it unreliable for MRD monitoring.

The aim of this PhD project is to evaluate the feasibility and reliability of an innovative method for the identification, quantification, and monitoring of AML-associated mutations based on the combination of NGS-driven dPCR. By combining NGS analysis, which provides a mutational gene profile at the time of diagnosis, with dPCR, a highly sensitive and accurate technique, we sought to define initial genetic risk and to quantify and monitor over time the gene mutations associated with leukemia clones identified by NGS.

The study design was conceived so that, at the time of diagnosis, biological samples (peripheral blood and bone marrow) would be collected from intensive chemotherapy candidate patients, with the aim of performing molecular analysis by NGS (myeloid panel, SOPHiA Genetics) and dPCR in order to identify the mutations present and then follow their progression over time by dPCR.

Pathological or potentially pathological mutations identified by NGS were subsequently monitored by dPCR on peripheral blood and bone marrow samples collected at different time points: after induction/consolidation therapy, at the end of treatment, and, in patients undergoing HSC transplantation, both before and after that procedure.

For some mutations, specific custom TaqMan assays were designed in the absence of commercially available probes for dPCR. The multiplex approach of dPCR allowed simultaneous analysis of multiple mutations, with significant savings in terms of time, cost, and reduced margin of error.

In addition, in case of relapse, it was always planned to collect peripheral blood and bone marrow samples for molecular re-evaluation by NGS to detect the possible appearance of new clones, and simultaneously by dPCR.

The feasibility of the study was defined as being able to monitor at least 70% of patients by dPCR. Preliminary results confirm the feasibility of the dPCR NGS-driven approach for MRD monitoring. Indeed, 86% of patients (12 out of 14) had at least one mutation identified by NGS at diagnosis, which was subsequently successfully monitored by dPCR. Most probes (14/18; 78%) were drawn. However, it is important to note that the initially high percentage of custom-designed probes could probably be reduced in the future by having already pre-designed probes available for the patients analyzed.

In addition, all selected mutations were detected in both bone marrow blood and peripheral blood samples. This finding, in the future, may suggest the possibility of monitoring MRD using only peripheral blood samples. Thus, the study achieved its main objective by demonstrating the feasibility of the combined dPCR NGS-driven approach for MRD monitoring in AML.

Compared to MRD assessment by traditional methods, the data obtained show two possible scenarios: (i) MRD monitoring in the absence of traditional markers, and (ii) MRD monitoring in the presence of traditional markers. In the latter case, the combined approach based on dPCR and NGS allowed not only to confirm the results obtained by traditional marker analysis, but also to anticipate molecular clearance and, in some cases, early detection or prediction of relapse.

In conclusion, the dPCR NGS-driven approach has been shown to be feasible and effective in identifying, quantifying, and monitoring disease-associated mutations, representing an advanced tool for MRD monitoring and more precise and personalized management of AML patients.

Index

1 – Acute Myeloid Leukemia (AML)

Acute Leukemias are a group of rare life-threatening haematopoietic disorders, characterized by the proliferation and differentiation of haematopoietic stem cells that have undergone malignant transformation[1].

Classification is based on evaluating the morphological characteristics of the cells in order to identify the best and clearly system for the accurate classification of a malignant clone as (i) Myeloid (ii) B Lymphoid (iii) T Lymphoid and (iv) Byphenotipic[1]. Depending on the occurrence of one or other of the above conditions, the leukemic population may have different phenotypical, morphological, immunological and/or molecular characteristics.

Of all Leukemia types, Acute Myeloid Leukemia (AML) is the most frequent type[2], and will be covered in detail in the following paragraphs.

Acute Myeloid Leukemia are malignant, biologically and clinically heterogeneous disease[3] of the haematopoietic system, that characterized by infiltration, at the level of Bone Marrow (BM), blood and other tissue (e.g., lymph nodes, spleen, liver, Central Nervous System [CNS] and testes[4]) by clonal haematopoietic cells with high proliferation, abnormal differentiation and, occasionally, poorly differentiation, called blasts cells (BC)[5], with low production of healthy hematopoietic cells[6]. The incidence is higher in adults and increases with age[7], with a median age at diagnosis of ≥ 68 years. The incidence rate rises exponentially, from 1.3 per 100,000 adults under 50 years old, to 5.1 in those aged 50–64, and 20.1 in those over 65 years[8]. Due to the increasing average age of the population, the incidence is expected to rise in the coming years[9].

The incidence is influenced not only by age but also by biological sex. According to the literature, it is higher in males[10-12]. In particular, N. Stabellini et al. reported that the incidence in males is approximately 1.4 times higher than in females, although the underlying reason remains unknown[11].

Besides age and gender, several risk factors have been identified: (i) pre-existing hematological diseases[13], (ii) prior chemotherapy treatment[13], (iii) genetic disorders and constitutional genetic defects[14], (iv) radiation exposure[13,15] (v) chemical or occupational hazards[16,17], (vi) infection[18] and (vii) viruses[16].

In most cases, the cause of the disease remains unknown, as malignancy arises de novo without any identifiable leukemogenic exposure. However, regarding the first, second, and fourth risk factors, "*Secondary Acute Myeloid Leukemia*" refers to AML developing in patients with a history of Myelodysplastic Syndromes (MDS), myeloproliferative

disorders[16], myelodysplastic/myeloproliferative overlap syndromes[19], prior chemotherapy, or confirmed exposure to leukemogenic agents[20]. These exposures include both therapeutic and non-therapeutic substances[16,19], such as cytotoxic drugs and radiation therapy, which are used for both neoplastic and non-neoplastic conditions[19]. Details on secondary AML are discussed in the Classification section. Additionally, symptoms like fatigue, bleeding, or recurrent infections occurring one month or more before AML diagnosis may suggest a preleukemic condition[20].

Although these factors are not direct causes of AML, they do suggest that the interplay between genetic predisposition, pre-existing conditions and environmental exposures can have a significant impact on the risk of developing the disease.

Lastly, the "*The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia*"[21], introduced a new nosological entity: familial acute leukemia and myelodysplastic syndrome. In particular, hereditary susceptibility includes telomere disorders due to mutations in TERC or TERT, familial platelet disorders and AML with mutations in *CEBPA*, *GATA2*[22] or *RUNX1*[18].

Etiology remains under investigation, with no definitive scientific certainties. However, it is evident that AML is a particularly complex and multifactorial disease, driven by the interplay of genetic, environmental, lifestyle factors, as well as pre-existing conditions. Notably, and only a small proportion of cases can be attributed to known risk factors[23].

A striking example of multifactoriality etiology is the ETV6-RUNX1 fusion gene: it is acquired in utero, but a secondary somatic mutation is required for its activation[18].

1.1 – Pathogenesis

Thanks to technological advances, particularly sequencing techniques, it is becoming increasingly possible to better understand the mechanisms underlying leukemogenesis and, more generally, disease mechanisms.

The first complete genome sequence of a patient with acute myeloid leukemia (M1 type, according to the FAB Classification) was reported in 2008[24]. From here, other Research Groups have sequenced AML cases, contributing to an initial characterization of the disease[25].

Based on the above, we can state with certainty that the onset and progression of AML required genetic events, such as the clonal expansion of Leukemic Stem Cell. Today, the predominant theory regarding AML – and the conceptual framework used to understand its

development – is the so-called "*two-hit hypothesis*"[26]. This hypothesis posits that at least two different genetic events (or "*hits*") are necessary for the initiation and progression of the disease. Specifically, these genetic alterations are categorized into two classes: (i) class I and (ii) class II mutations[26,27].

Class I mutations do not affect hematopoietic differentiation but confer a proliferative and survival advantage to cells. In contrast, class II mutations primarily affect hematopoietic differentiation, with only a modest impact on the proliferation and survival rate[26].

Therefore, AML can be considered the result of the cooperation between two classes of oncoproteins: class I and class II, leading to aberrant differentiation, enhanced cell survival, and increased proliferation[26]. However, a key limitation of this model is the absence of identifiable class I and class II mutations in the majority of AML cases[27]. Despite this, indirect evidence supporting the model comes from the mutual exclusivity of intraclass mutations[27]. In fact, reports of AML cases harboring more than one class I mutation are extremely rare[28-30].

Moreover, leukemia-associated genetic aberrations, such as Core Binding Factor (CBF) translocations, are found not only in patients with AML but also in healthy individuals[31]. This suggests that a single mutation alone is insufficient to drive leukemic progression.

Class I and class II mutations are listed in Table 1.

Class I mutations	**Class II mutations**
BCR–ABL	CBF*β*–MYH11
N-RAS	AML1–ETO
K-RAS	TEL–AML1
c-KIT (exon 8)	PML–RAR*α*
c-KIT (Asp 816)	NUP98–HOXA9
FLT3 (ITD)	PU.1
FLT3 (Asp 835)	C/CEP*α*
PTPN11	AML1
NF1	AML1–AMP19
TEL- PDGFRβ	
AML1–COPINE VIII	

Table 1: List of class I and class II mutations, as described by Reilly J.T. [27].

1.2 – Symptoms and clinical course

The clinical presentation of AML is highly variable, generally falling into two distinct categories: (i) asymptomatic patients with abnormalities in one or more blood tests and (ii) patients with severe, clinically overt disease, sometimes requiring Intensive Care Unit (ICU) support[32].

Initial manifestations are generally non-specific and gradually become more pronounced as leukemic blasts infiltrate the bone marrow. In most cases, patients present with symptoms related to bone marrow failure and abnormalities Complete Blood Count (CBC).

In the context of bone marrow failure, cytopenia-related symptoms are among the primary clinical manifestation of AML. These include: (i) anemia-related symptoms (e.g., asthenia, paleness, fatigue and dyspnea), (ii) thrombocytopenia-related symptoms (e.g., ecchymosis, purpuric, and other hemorrhagic manifestations such as gingival bleeding and epistaxis) and (iii) neutropenia-related symptoms (e.g., infections). Conversely, an abnormal complete blood count may present as cytopenia, leukocytosis and/or circulating blasts[33].

In addition, at the time of diagnosis, organ infiltration (e.g., lymph nodes, skin) may occur, along with Central Nervous System involvement[33], which has an incidence between 1.7 and 5.06%[34].

Although rare, with an estimated incidence of 2.5-9.1%[35] among all patients with AML, myeloid sarcoma may also occur[33].

As reported, extramedullary localization (EML) of AML, can be observed[36]. EML may precede bone marrow involvement or occur in patients who are in Complete Remission (CR) according to hematological and bone marrow evaluations.

1.3 – Diagnosis

To diagnose AML, identify its subtype, and rule out other differential diagnoses, several tests are required. First, an anamnestic assessment of the patient, including medical history and thorough physical examination, is necessary. If suspicion is confirmed, a complete blood count and differential count, along with morphological analysis (both peripheral blood and bone marrow), immunophenotypic, cytogenetic, and molecular mutation analyses are performed to define molecular signatures.

As previously reported, after diagnosing AML, the next step is classifying it into a subtype, for risk stratification, prognosis and to determinate the best possible treatment.

To date, bone marrow aspirate remains the "*gold standard*", for morphological assessment. At the bone marrow level, both blast and non-blast cells (e.g., basophil, erythroid precursor, promonocytes and atypical promyelocytes) can be identified, and dysplasia is also evaluated. The blast cells count is performed alongside the morphological examination. In addition to aiding diagnosis and differential diagnosis, the number of blast cells serves as an indicator of disease progression.

Molecular genetic analysis must be conducted alongside morphological analysis. Cytochemistry, which aids in diagnosing and classifying AML, has been partially superseded by immunophenotyping[37].

Immunophenotyping is performed in all cases with a clear suspicion of AML, using Flow Cytometry (FC) or, alternatively, immunohistochemistry (IHC)[38], with bone marrow being the preferred sample. This helps define the cell lineage and determine the AML sub-category[39,40]. Multiparameter FC analysis with marker CD45/side scatter gating is preferred due to the large number of antigens that can be studied[37]. Immature markers typically expressed include CD34, CD117 and HLA-DR, while myeloid and granulocytic markers include myeloperoxidase (MPO), CD13, CD15 and CD33.

AML it is confirmed when at least two of the following markers are expressed by myeloblasts: MPO, CD13, CD33, CDw65 and CD117[41].

Approximately 25% of AML patients express detectable lymphoid lineage antigens. In particular, CD7, a T-cells antigen, and CD19, a B-cells antigen, are presents in approximately 10-30% and 3% of AML cases, respectively[41].

In some cases, we encounter *"biphenotypic"* AML, where both myeloid and lymphoid marker are prominently present[40,41].

This analysis not only shows the blast count but also provides insights into underlying mutations, cytogenetic abnormalities and prognosis. For example, myeloblast that express CD34 and have mutations in NPM1 gene indicate a poor prognosis[42].

Immunohistochemistry can be used as an alternative to flow cytometry when bone marrow samples are inadequate (e.g., due to fibrosis). CD34 is the most commonly used marker, followed by CD117, CD33, CD71, MPO and CD61[43].

As mentioned earlier, the evaluation of the karyotype is crucial for diagnosing AML, particularly since translocations, inversions, deletions or additions are common in these patients.

In addition to conventional karyotyping, Fluorescent in Situ Hybridization (FISH) analysis can be performed[37], utilizing fluorescent probes targeting specific chromosome loci[44]. FISH

is particularly useful for confirming chromosomal abnormalities detected by classic cytogenetics, as well as for identifying cryptic deletions and subtle translocations, such as inv(16)(p13.1q22), PML-RARA and t(9;22)[45].

The importance of cytogenetic analysis extends beyond diagnosis to include prognosis[37] and the selection of the best therapeutic approach for the patient.

Molecular analysis has become increasingly important in AML, where high variability is observed, especially in recent years. It is crucial not only for classification but also for prognostication (especially when a normal karyotype is found[37]), therapeutic decision and Measurable Residual Disease (MRD) monitoring[44]. Multiple techniques can be used in both diagnostic and research settings, including (i) reverse transcription polymerase chain reaction (RT-qPCR) (ii) Sanger sequencing, (iii) Next Generation Sequencing (NGS), (iv) Whole Genome Sequencing (WGS) (v) Whole Exome Sequencing (WES) and (vi) Whole Transcriptome Sequencing (WTS)[44].

In NGS analysis, standard gene panels are typically used (e.g., SOPHiA DDM™ Dx Myeloid Solution). NGS provide detailed information, including the gene, type of mutation, and category of mutation, allowing for the distinction between pathological and non-pathological mutations. As reported, molecular analysis is crucial for classification, prognostication, therapeutic decision and MRD monitoring. From classification perspective, molecular analysis can distinguish between "*de novo*" AML and "*secondary*" AML. Specifically, involved in chromatin remodeling and splicing are more commonly associated with secondary AML[46].

1.4 Classification

As we have seen, multiple examinations play a crucial role in the diagnostic process and classification of crucial for the classification of AML, which has undergone significant revisions over the years.

The classification of AML is essential for prognosis and treatment planning. The main classification systems include:

- FAB (French-American-British) classification.
- WHO (World Health Organization) classification.
- ICC (International Consensus Classification) classification.

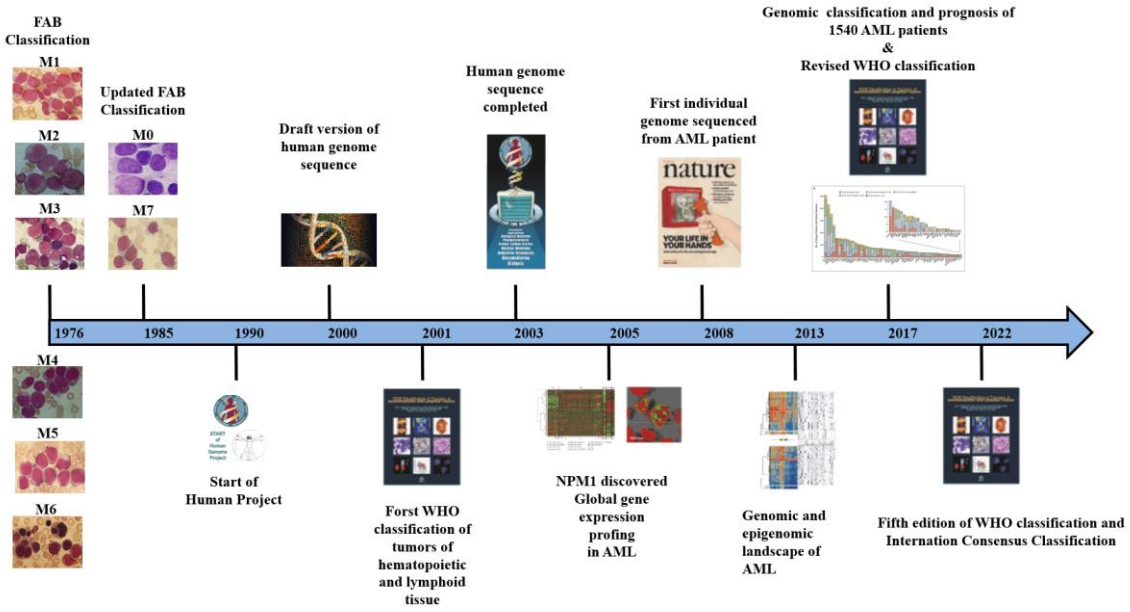

Figure 1: Chronological evolution of AML classification.

1.4.1 – FAB classification

The FAB classification (French-American-British), was the first system established for AML classification, based exclusively on morphology, levels of differentiation between the different cell lines, and the degree of cell maturation.

The first proposal for a classification system can be traced back to work of Bennet J.M. et al.[39], where a group of hematologists convened to outline key features and define a "*universal*" nomenclature.

The primary goal of the Group was to classify the neoplasms as myeloid or non-myeloid and then establish criteria for defining subgroups based on morphology.

The FAB classification defines six distinct subgroups, M1 through M6, based on the direction of differentiation and the degree of maturation: (i) Myeloblastic leukemia without maturation, (ii) Myeloblastic leukemia with maturation, (iii) Hypergranular promyelocytic leukemia, (iv) Myelornonocytic leukemia, (v) Monocytic leukemia and (vi) Erythroleukemia[39].

M1, M2 and M3 are characterized predominantly by granulocytes but each with different degrees and types of maturation, M4 subgroup is composed mainly of granulocytes and monocytes, the M5 subgroup of monocytes and the M6 subgroup of erythroblasts.

Over the years, the FAB classification has been expanded, with M7 (*Acute Megakaryoblastic Leukemia*) and M0 (*Acute Myeloid Leukemias with Minimal Differentiation*) subgroup added

in 1985 and 1991, respectively[47]. These updated incorporated immunophenotyping and electron microscopy studies[48,49].

Briefly, the table below (*Table 2*) shows the nosocomial entities present in the FAB classification after the initial release in 1976 and the updates in 1985 and 1991.

FAB subtype	**Name**
M0	Acute Myeloid Leukemias with Minimal Differentiation
M1	Myeloblastic leukaemia without maturation
M2	Myeloblastic leukaemia with rnaturation
M3	Hypergranular promyelocytic leirkaemia
M4	Myelornonocytic leukaerniu
M5 A B	Monocytic Ieukaemia Poorly differentiated Differentiated
M6	Erythroleukaemia
M7	Acute Megakaryoblastic Leukemia

Table 2: Different subtypes of FAB classification.

1.4.2 – World Health Organization (*WHO*) 2022 classification

Technological advances, particularly in molecular biology, have necessitated revisions to the classification of AML, integrating morphology (cytology and histology), cytogenetics, immunophenotyping, and molecular markers[50].

The World Health Organization (*WHO*) introduced a new classification system in 1995, which has since been updated and revised in 2001, 2008, 2016 and 2022. The fifth edition, currently in use, categorizes AML into three distinct groups: (i) Acute Myeloid Leukemia with defining genetic abnormalities, (ii) Acute Myeloid Leukemia, defined by differentiation, and (iii) Myeloid Sarcoma[51] (*Table 3*).

Acute Myeloid Leukemia with defining genetic abnormalities
Acute promyelocytic leukemia with PML::RARA fusion
Acute myeloid leukemia with RUNX1::RUNX1T1 fusion
Acute myeloid leukemia with CBFB::MYH11 fusion

Acute myeloid leukemia with DEK::NUP214 fusion
Acute myeloid leukemia with RBM15::MRTFA fusion
Acute myeloid leukemia with BCR::ABL1 fusion
Acute myeloid leukemia with KMT2A rearrangement
Acute myeloid leukemia with MECOM rearrangement
Acute myeloid leukemia with NUP98 rearrangement
Acute myeloid leukemia with NPM1 mutation
Acute myeloid leukemia with CEBPA mutation
Acute myeloid leukemia, myelodysplasia-related
Acute myeloid leukemia with other defined genetic alterations
Acute myeloid leukemia, defined by differentiation
Acute myeloid leukemia with minimal differentiation
Acute myeloid leukemia without maturation
Acute myeloid leukemia with maturation
Acute basophilic leukemia
Acute myelomonocytic leukemia
Acute monocytic leukemia
Acute erythroid leukemia
Acute megakaryoblastic leukemia

Table 3: Acute Myeloid Leukemia, 5th WHO Classification. Adapted from Khoury J.D. et al.[51].

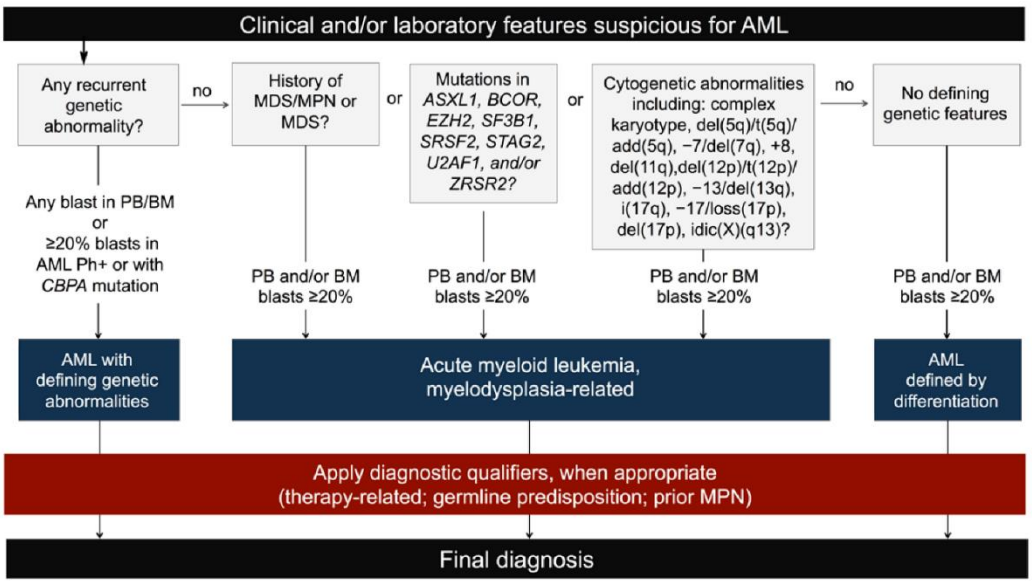

Figure 2: WHO diagnostic flowchart. From Pizzi et al.[52].

1.4.3 – International Consensus Classification (*ICC*) 2022

Finally, based on the 4th edition of the WHO classification, a group of experts collaborated on the creation and publication of International Consensus Classification (ICC). This system employs a multiparametric approach that integrates clinical and biological features for a more comprehensive diagnostic evaluation[53]. In contrast to the 5th edition of the WHO classification, the ICC retrains the 20% blast threshold for most cases. The key difference is that, except for "*AML with recurrent genetic abnormalities*", a blast percentage greater than 20% in required to make a diagnosis of AML. However, when the percentage of blast is between 10 and 19%, it indicates an MDS/AML situation, representing a diagnostic continuum between MDS and AML. This range highlights the potential heterogeneity and overlap between the two conditions, helping to refine diagnosis and therapeutic strategies[53]. Following a hierarchical structure, the ICC classification outlines five groups: (i) AML with recurrent genetic abnormalities, (ii) AML with mutated *TP53*, (iii) AML with MDS-related mutations, (iv) AML with MDS-related cytogenetic alterations and (v) AML not otherwise specify (NOS)[53]. In the figure below (*Figure 3*) the diagnostic flowchart is reported.

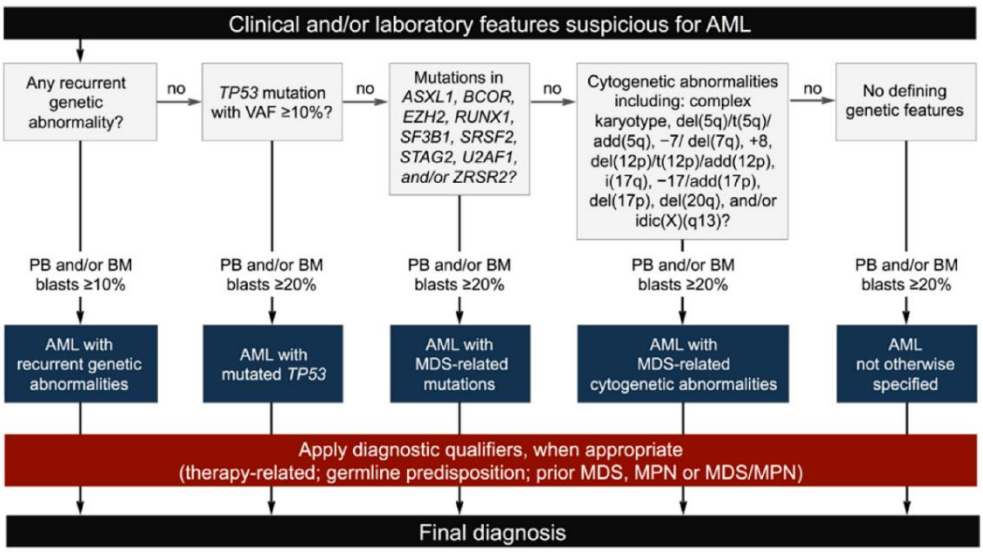

Figure 3: ICC diagnostic flowchart. The flowchart follows a hierarchical decision-making process from left to right. From Pizzi et al.[52].

As previously mentioned, "*AML with recurrent genetic abnormalities"*, does not require the ≥20% blast threshold. In the table below (*Table 4*), the recurrent genetic abnormalities are listed[53].

AML with recurrent genetic abnormalities
Acute promyelocytic leukemia (APL) with t(15;17)(q24.1;q21.2)/PML::RARA ≥ 10%
APL with other RARA rearrangements* ≥ 10%
AML with t(8;21)(q22;q22.1)/RUNX1::RUNX1T1 ≥ 10%
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22)/CBFB::MYH11 ≥ 10%
AML with t(9;11)(p21.3;q23.3)/MLLT3::KMT2A ≥ 10%
AML with other KMT2A rearrangements** ≥ 10%
AML with t(6;9)(p22.3;q34.1)/DEK::NUP214 ≥ 10%
AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2)/GATA2; MECOM(EVI1) ≥ 10%
AML with other MECOM rearrangements*** ≥ 10%
AML with other rare recurring translocations ≥ 10%
AML with t(9;22)(q34.1;q11.2)/BCR::ABL1**** ≥ 20%
AML with mutated NPM1 ≥ 10%
AML with in-frame bZIP CEBPA mutations ≥ 10%

Table 4: AML with recurrent genetic abnormalities.

** Includes AMLs with t(1;17)(q42.3;q21.2)/IRF2BP2::RARA; t(5;17)(q35.1;q21.2)/NPM1::RARA; t(11;17)(q23.2;q21.2)/ZBTB16::RARA; cryptic inv(17q) or del(17) (q21.2q21.2)/STAT5B::RARA, STAT3::RARA; Other gens genes rarely rearranged with RARA:TBL1XR1 (3q26.3), FIP1L1 (4q12), BCOR (Xp11.4).*

***Includes AMLs with t(4;11)(q21.3;q23.3)/AFF1::KMT2A#; t(6;11)(q27;q23.3)/AFDN::KMT2A; t(10;11)(p12.3;q23.3)/MLLT10::KMT2A; t(10;11)(q21.3;q23.3)/TET1::KMT2A; t(11;19)(q23.3;p13.1)/KMT2A::ELL; t(11;19)(q23.3;p13.3)/KMT2A::MLLT1 (occurs predominantly in infants and children).*

****Includes AMLs with t(2;3)(p11∼23;q26.2)/MECOM::?; t(3;8)(q26.2;q24.2)/MYC, MECOM; t(3;12)(q26.2;p13.2)/ETV6::MECOM; t(3;21)(q26.2;q22.1)/MECOM::RUNX1.*

*****The category of MDS/AML will not be used for AML with BCR::ABL1 due to its overlap with progression of CML, BCR::ABL1-positive. From Arber D.A.*[53].

ICC and WHO Classification are currently the most commonly used system for classifying AML. While both the latest editions of the classification share the same framework, they differ in several significant ways. Notably, the WHO Classification does not include AML with *TP53* mutations such as independent nosological entity[52,54,55], even though mutations in *TP53* gene are recognized as important prognostic marker[52].

1.5 – Prognosis and prognostic indexes: risk stratification

In 2022, the European Leukemia Net (ELN) published an important paper with updated recommendations, including a comprehensive risk stratification system[56].

The classification of AML subtype is crucial not only for diagnosis but also for determining treatment strategies, prognosis and follow-up after therapy initiation, such as monitoring MRD.

The ELN risk stratification system identifies three distinct risk classes based on molecular and cytogenetic features: (i) favorable, (ii) intermediate and (iii) adverse[56]. Further details regarding this stratification can be found in *Table 5*.

Generally, favorable-risk patients are associated with a relatively good prognosis, showing a favorable response to standard chemotherapy regimens and a higher likelihood of achieving long-term remission. In contrast, patients with intermediate-risk status have a variable prognosis, which can depend on other prognostic factors such as age and response to therapy. Lastly, patients classified as adverse risk typically have a poor prognosis, and they are often considered for early allogenic stem cells transplantation[56].

Risk category	**Genetic abnormalities**
Favorable	t(8;21) (q22;q22.1); RUNX1-RUNX1T1
	inv (16) (p13.1q22) or t (16;16) (p13.1;q22); CBFB-MYH11
	Mutated *NPM1* without FLT3-ITD
	bZIP in-frame mutated *CEBPA*
Intermediate	Mutated *NPM1* with FLT3-ITD
	Wild-type *NPM1* with FLT3-ITD
	t (9;11) (p21.3;q23.3); MLLT3-KMT2A
	Cytogenetic abnormalities not classified as favorable or adverse
Adverse	t (6;9) (p23;q34.1); DEK-NUP214
	t(v;11q23.3); KMT2A rearranged
	t (9;22) (q34.1;q11.2); BCR-ABL1
	inv(3) (q21.3q26.2) or t (3;3) (q21.3;q26.2); GATA2, MECOM(EVI1)

	t (3q26.2;v); MECOM (EVI1)-rearranged
	−5 or del (5q); −7; −17/abn (17p)
	Complex karyotype, monosomal karyotype
	Mutated *ASXL1, BCOR, EZH2, RUNX1, SF3B1, SRSF2, STAG2, U2AF1, or ZRSR2*
	Mutated *TP53*

Table 5: Risk stratification according to European Leukemia Net. Adapted from Döhner H. et al.[56].

1.6 – Biological features

The previous sections emphasized the importance of cytogenetic and molecular alterations in AML. The advent of Next-Generation Sequencing techniques has enabled the identification of 40-50 genes with recurrent somatic mutations, which are commonly associated with various AML subtype[57-60]. In particular, genes that play a putative role in AML pathogenesis – either through mutational events and/or chromosomal aberration - are categorized into different groups[58,61] (*Figure 4*).

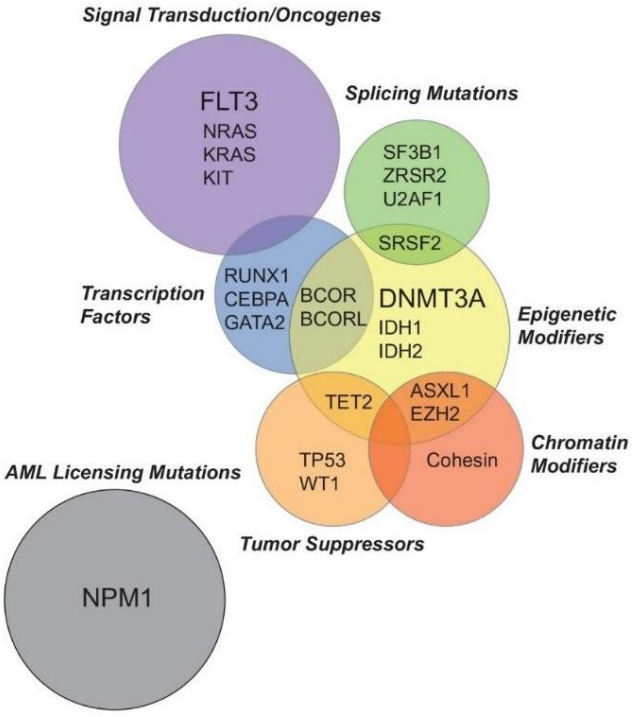

Figure 4: Driver mutations in AML. The figure above shows the functional overlap of driver mutations, stratified by their mechanistic consequence. A larger circle size corresponds to a higher frequency of events. From Kishtagari A. et al.[58].

Papaemmanuil E. and colleagues, in one of the largest studies of AML involving 1,540 patients, identified 5,234 driver mutations across 76 genes or genomic regions, highlighting the complexity of this asset. At least one mutation was found in 1,478 of 1,540 (96%), and two or more mutations were found in approximately 86% of the cases[57].

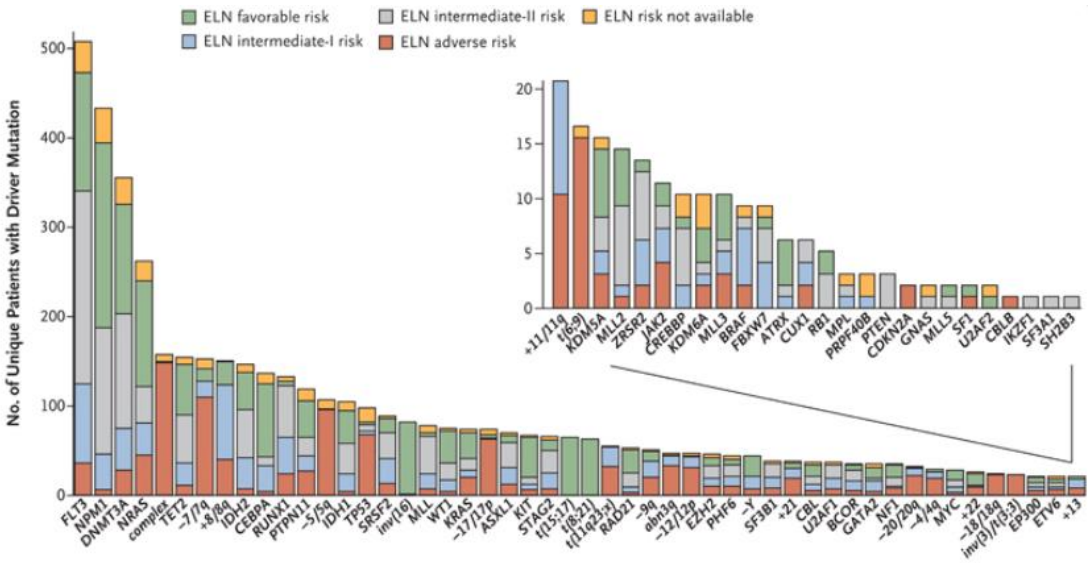

Figure 5: Landscape of Driver Mutations in AML. This study included1,540 AML patients. Different colors in bars represent the molecular risk according to the ELN classification. From Papaemmanuil E. et al.[57].

Another study[59] conducted by Patel J.P. and colleagues highlighted the complexity of AML in terms of genetic profile, as also shown by Papaemmanuil E et al[57]. In this work, 504 patients were evaluated, whit 398 in the test cohort and 104 in the validation cohort. The mutational status was assessed from bone marrow and peripheral blood samples in 277 (55.2%) and 225 (44.8%) of 502 cases, respectively. In 97.3% of patients, at least one somatic mutation was identified.

The figure below (*Figure 6*) shows the frequency of gene mutations and the interrelationships among the various mutations. As noted, both *FLT3-ITD* and *FLT3-TKD* exhibit the highest mutational frequencies, followed by *NPM1* and *DNMT3A*, with 37%, 29%, and 23%, respectively.

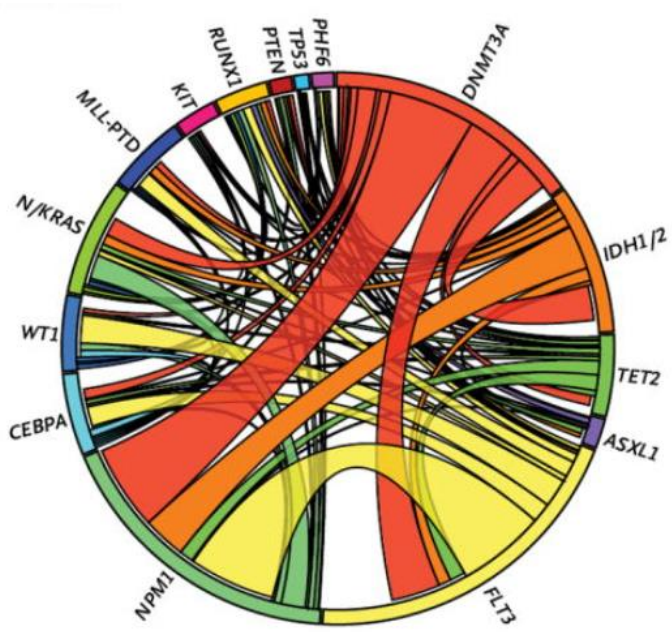

Figure 6: AML mutational complexity. Circle plot showing the frequency of gene mutations and the interrelationships among the various mutations. From left to right (clockwise), the length represents the frequency of mutations in the first gene, while the width represents the percentage of patients who also have a mutation in the second age. Adapted from Patel J.P. et al.[59].

1.7 – Therapy

The treatment of AML is typically divided into three phases: (i) the induction phase, (ii) the consolidation phase and (iii) the maintenance phase.

In the induction phase, the goal is to achieve Complete Response (CR), Complete Remission with incomplete hematologic recovery (CRi) or Morphologic Leukemia Free State (MFLS). However, achieving CR (or CRi, or MFLS) does not necessarily mean complete elimination of the disease. A small portion of leukemia cells may remain in the body, leading to the presence of Measurable Residual Disease.

This brings us to the second phase: consolidation. During this phase, aggressive chemotherapy is administered, followed, in some cases, by autologous or allogenic stem cell transplantation.

Classic induction therapy, known as 3+7, consists of administering anthracyclines (such as Daunomycin, Doxorubicin, Idarubicin and Mitoxantrone) for three days, in combination with Cytarabine (ara-C) for 7 days[56,62]. Additionally, it has become standard practice to add a Kinase Inhibitor (KI) in the first line of treatment for patients with *FLT3* mutations. The

combination of chemotherapy with a Kinase Inhibitor has been shown to prolong both Overall Survival (OS) and Event Free Survival (EFS)[63].

The consolidation phase follows the achievement of CR, CRi or MFLS and, generally, and is typically carried out with Cytarabine. In some Centers, high dose Cytarabine (3000 mg/m^2), is still used, which increases toxicity without improving survival outcomes. In contrast, other Centers administer intermediate dose of Cytarabine, which may reduce toxicity while still providing effective treatment[64-67].

Consolidation can also be represented, as mentioned, by transplantation and it is recommended for patients with 35-40% of relapse risk[68], for patients with adverse risk and non-adverse risk and MRD persistence[56].

Autologous Stem Cells Transplantation can be utilized in patients with favorable or intermediate risk with adequate MRD response or for patients for whom allogeneic transplantation is not a viable option[69].

The maintenance phase is defined by the FDA as a phase in which, once CR has been achieved, a further course of treatment, typically time-limited and less toxic, is administered to reduce the risk of relapse.

In the case of resistance to induction therapy or relapses, second-line treatment can be considered. If a second CR is achieved and the patients' age and overall conditions permit, allo-SCT is strongly recommended[70].

In addition, several studies are underway exploring chemo-free approaches, particularly for elderly patients and those with multiple comorbidities.

Lastly, innovative approaches in AML treatment include immunotherapy-based strategies, such as Bispecific Antibodies (BsAbs) and Chimeric antigen receptor (CAR) T-cell therapy.

1.8 – Response criteria

Treatment response criteria for Acute Myeloid Leukemia are essential for evaluating how effectively a patient responds to therapy. These criteria are standardized and follow guidelines proposed by the European Leukemia Net[71] and International Working Group (IWG)[20] for AML. The response groups response groups include: (i) Complete Remission, (ii) Complete Remission with Incomplete Hematologic Recovery (CRi), (iii) Partial Remission (PR), (iv) Morphologic Leukemia-free State (MLFS), (v) relapse and (vi) Treatment Failure.

Complete remission (CR) is defined by several specific criteria. The bone marrow blast percentage must be less than 5%. At peripheral blood examination, the presence of circulating blast or blast with Auer rods is not permitted. The Absolute Neutrophil Count (ANC) and platelet count should be greater than $1.0*10^9$/L and $100*10^9$/L, respectively. The patient must be transfusion-independent, although hemoglobin levels and hematocrit are not considered to define CR status. Additionally, there must be no evidence of disease, as defined by the absence of AML in peripheral blood and no evidence of extramedullary disease (e.g., soft tissue or central nervous system involvement). Finally, there must be an initial normalization of blood cell production in the bone marrow.

The "*Complete Remission with Incomplete Hematologic Recovery*" is a response status that differs from CR for hematological recovery. In CRi, the Absolute Neutrophil Count and platelet count should be less than $1.0*10^9$/L and $100*10^9$/L, respectively.

In the "*partial remission*" state, there must be a 5-25% reduction of blasts in the bone marrow if the initial blast value at onset was between 50 and 100%. Vice versa, if the blast percentage at onset was between 20 and 49%, a decrease a decreased of at least half is required, while still maintaining a value above 5%. There must be no evidence of extramedullary disease, and blood counts should improve but not yet normalized. If Auer rods are present, a value of $\leq$ 5% blasts may also be considered PR.

In the "*Morphologic Leukemia-free State*" the patient has bone marrow blast less than 5% with a count of at least 200 nucleated cells. There must be an absence of extramedullary disease, and no blasts with Auer rods must be found. In this state, peripheral blood examination does not meet the criteria for be defined CR or PR.

Regarding CR, CRi, PR and MLFS, the 2022 ELN recommendations removed the criterion of "not having blasted with Auer rods"[56].

Relapses are defined as return of 5% or more blasts in bone marrow after achieving CR, with a simultaneous reappearance of blasts in the peripheral blood. In some cases, the reappearance of AML outside the bone marrow is also considered a relapse. The percentage of blasts in the bone marrow must not be attributable to other causes (e.g., regeneration after therapy).

Lastly, the "*treatment failure"* state can be divided into three different subgroups: (a) persistent disease, (b) stable disease and (c) progressive disease. Persistent disease is defined as the failure to achieve CR or PR after treatment. Stable disease refers to an absence of change in terms of blasts percentage or blood counts following treatment. Progressive

disease, however, is characterized by an increase in blasts percentage in the bone marrow, peripheral blood or booth or the development of new extramedullary disease.

It is also very important to evaluate Measurable Residual Disease, in patients who are in complete morphological remission, as there are two distinct scenarios: (i) MRD negative and (ii) MRD positive.

MRD negative means that there are no detectable leukemia cells using sensitive techniques, such as flow cytometry or PCR, beyond what can be seen under the microscope. On the other hand, MRD positive refers to detection of leukemic cells despite the patient being in morphologic CR. As a result, the response terminology is extended to include MRD status (e.g., CR, CRi and CRh without MRD, respectively, CR_{MRD-}, CRi_{MRD-}, CRh_{MRD-}).

A third scenario involves a patient in complete morphological remission but with low-level molecular MRD detection, such as qPCR for NPM1 <2%[72].

2 – Measurable Residual Disease (MRD)

In the field of onco-hematology, measurable residual disease (MRD) is defined as the presence of residual leukemic cells in a patient after treatment. Considering that a 70 kg adult subject has approximately from 10 to 13 per 10^9 nucleated bone marrow cells per kg[73], and the CR is defined by a threshold of less than 5% of myeloblast, it is estimated that a patient in CR may still harbor between 10^{10} to 10^{12} leukemia cells[74]. This occurs despite a significant reduction in disease burden - typically 99.9% in most cases[75]. This highlights the limitations of conventional remission criteria and underscores the importance of MRD assessment in refining prognosis and guiding therapeutic strategies, especially since approximately 60-70% of patients defined in CR eventually relapsed[76].

In addition, more than 30% MRD negative patients have experienced relapse[77], highlighting the need for continuous research in this area.

MRD evaluation is complicated by the low proportion of leukemic cells relative to other blood cells. For instance, the ratio between white blood cells and leukemic cells ranges from $1:10^4$ to $1:10^6$ [78], highlighting the necessity for highly sensitive detection techniques.

In the figure below (*Figure 7*) the concept of MRD is summarized, showing the main scenarios that can occur. As noted, the risk of relapse increases as the number of residual leukemia cells rises. Patients following the A line (persistent disease), can be evaluated with microscopy. Patients with a higher disease burden (B and C lines) have higher relapse incidence rate (very early and early relapse, respectively) and less disease-free survival compared to patients with a lower disease burden (D line) or those with no disease burden (E line). In some cases, leukemic cells can be completely eradicated (F line)[79]. Achieving a level of 10^{-6} or lower is considered deep remission[80].

MRD relapse is defined as the conversion from MRD negativity to MRD positivity, regardless of the technique used for evaluation (this conversion must be confirmed within four weeks, preferably whit a bone marrow sample). Alternatively, relapses can also be identified by increased in MRD levels of more than 1 $\log_{10}$ between two positive samples measured in the same tissue[72].

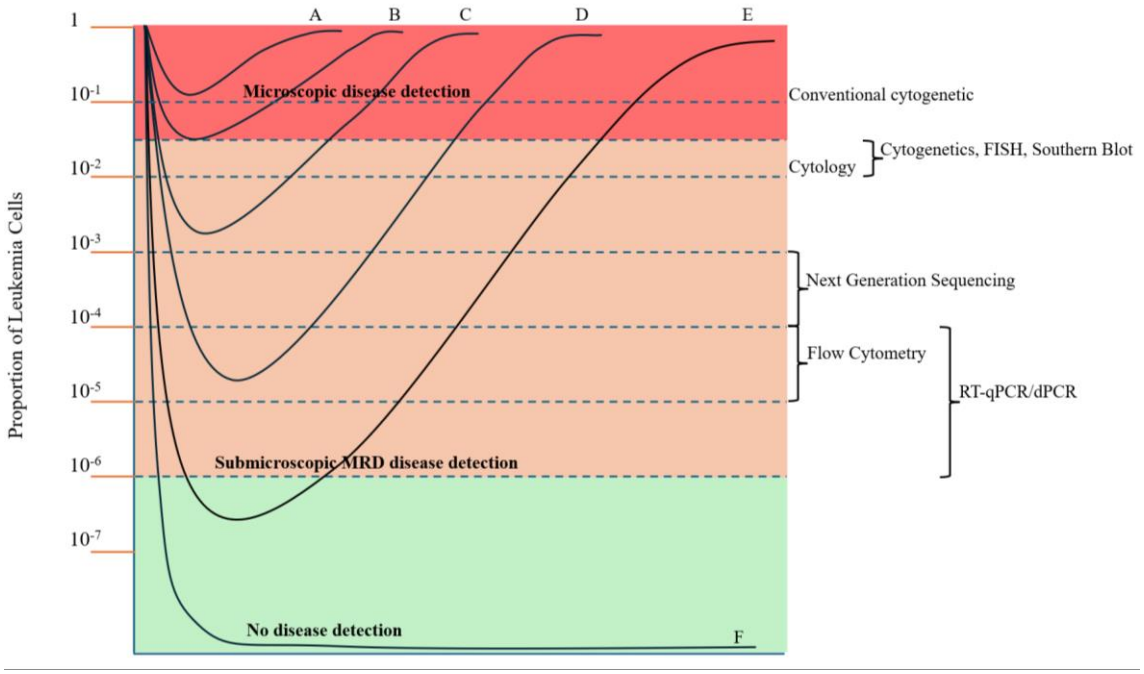

Figure 7: concept of MRD. A line indicated persistence of disease and can be detectable with microscopy, B and C lines represent patients with a high disease burden where relapses can occur prematurely, D and E lines represented patients with a low, or even absent, disease burden, in this case the relapsed may occur late. Lastly, F line shows the case in which leukemic cells can be totally eradicated. FISH: Fluorescent In Situ Hybridization; RT–PCR: reverse transcriptase PCR; dPCR: Digital PCR. Adapted from Buckley S.A. et al.[79].

A recent systemic review of the literature by Short N.J. et al.[81], analyzed 81 clinical studies involving over 11,500 patients. The study highlights the significant impact of MRD status on long-term outcomes in AML. MRD^- patients exhibit a significantly higher 5-year disease-free survival rate (DFS) compared to MRD^+ patients, with rates of 64% and 25%, respectively. In addition to DFS, the overall survival rate is also higher in MRD^- patients compared to MRD^+ patients, whit survival rates of 68% versus 34%, respectively. These striking results are illustrated in figure 8, underscoring the prognostic significance of MRD status in AML.

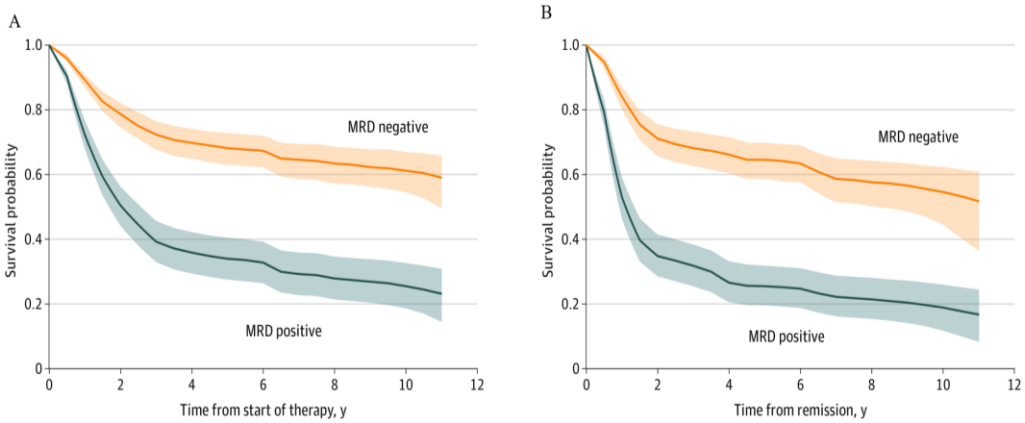

Figure 8: overall survival rate (A) and disease-free survival rate (B) based on MRD status. Adapted from Short N.J. et al.[81].

Of particular importance is MRD evaluation before transplantation, at it serves as a strong predictor of transplant outcome[82]. Additionally, MRD positivity pre-transplant may influence donor selection, favoring haploidentical donor over an HLA-matched sibling[83] and may also to the intensity of conditioning regimens. However, there are conflicting opinions, with some studies suggest there is no significant correlation[84], while others suggest a clear association[85].

MRD evaluation must be conducted with a higher sensitivity threshold than conventional cytology (approximately higher than 10^{-2}). To achieve this level of sensitivity and to detect up to a single pathologic cell in $10*10^4$ or 10^5 normal cells[86], the main techniques used are (i) Reverse Transcriptase-Quantitative Polymerase Chain Reaction, (ii) Multiparametric Flow Cytometry (MFC), (iii) Next Generation Sequencing and (iv) digital Polymerase Chain Reaction[82].

Briefly, in the table below (*Table 6*) the main techniques for monitoring MRD and their sensitivity limits are given.

Method of detection	**Sensitivity**
Conventional cytogenetic	10^{-1}
Cytology	10^{-2}
RT-qPCR	10^{-4} to10^{-6}
MFC	10^{-4} to10^{-5}
NGS for single nucleotides variants	10^{-3} to10^{-4}
dPCR	10^{-4} to10^{-6}

Table 6: The main techniques for the monitoring of MRD and their limits of sensitivity.

In addition to the sensitivity, the Limits of Detection (LOD) and Limit of Quantification (LOQ) are also very important factors. The LOD represents the smallest measurable signal, while the LOQ indicates the smallest value of a measurement that can be provided with an acceptable level of reliability and known uncertainty[87].

Reverse Transcriptase-Quantitative Polymerase Chain Reaction is a widely used technique in many laboratories due to its reproducible, speed and ability to provide quantitative results with standardization of housekeeping genes to avoid false negatives. As one of the most validated methods for monitoring MRD, it offers a sensitivity ranging from 10^{-4} to 10^{-6} [88]. In fact, qPCR-based technology is considered the "gold standard" for molecular MRD monitoring[89].

Another specific method that can be used in more than 90% of AML cases is Multiparametric Flow Cytometry (MFC), which has a sensibility range from 10^{-4} to10^{-5} [88]. This system is based on combining antigens and/or physical abnormalities found in leukemic cells, such as Leukemia-associated immunophenotypes (LAIP)[88,90] and the Different from Normal (DfN) approach, which is defined by an aberrant leukemic phenotype compared to healthy cells. Generally, most studies suggest a cutoff of 0.1%, which is recommended for distinguishing between MRD-positive and MRD-negative patients[78].

The Next Generation Sequencing approach is relatively new and certainly deserves to be studied and deepened and started to replace sanger sequencing[89]. Monitoring MRD with the NGS approach can follow three different paths: (i) Whole Genome Sequencing, (ii) Whole Exome Sequencing and (iii) targeted sequencing. In these methods, there is no specific molecular marker that can be followed by qPCR. Instead, all mutations can be tracked simultaneously, providing information into clonal evolution[91,92]. This approach allows for the identification of both leukemic clones and distinct subclones based on VAF, whit high VAF indicating leukemic clones and low VAF indicating subclones[91,93,94].

Furthermore, distinguishing between clonal mutations associated with hematopoiesis and those related to AML is another major challenge[9]. Somatic mutations can be present in the absence of hematological malignancy (known as clonal hematopoiesis of indeterminate potential), and their presence and persistence may be observed in a CR state, but they are not considered prognostic[95]. Jongen-Lavrencic M. and colleagues analyzed 482 patients, of which 430 (89.2%) showed at least one mutation. Their study specified that mutations in DTA genes (*DNMT3A, TET2* and *ASXL1*) did not have prognostic value, a finding also confirmed by Heuser M. et al.[96].

The latest evidence suggests that the combination of MFC and NGS technique represents a reliable combination for MRD detection, helping to implement and overcome the limitations of NGS[95,97].

The technique that is becoming increasingly used for MRD monitoring is digital Polymerase Chain Reaction, known for its high sensitivity, accuracy and reproducibility. Several studies in recent years have explored its potential applications. Compared to the NGS approach, dPCR has demonstrated a higher sensitivity, detecting variant alleles at a frequency as low as 0.002%. It can also identify very rare cells, as few as 1 in 15.000[98]. However, the main limitation of this technique, like RT-qPCR, is its highly specificity, which requires known genetic abnormalities.

In addition to the methods previously mentioned, other systems for monitoring MRD are still under development. These include microfluidic system, LNA-qPCR and Raman spectroscopy (and it's variant Surface-Enhanced Raman Scattering - SERS)[75].

In recent years there has been a significant effort to standardize monitoring techniques, establish positivity thresholds, define the optimal timing for evaluation and determinate the best starting material. The 2022 ELN guidelines recommend specific timepoints for assessing MRD: (i) diagnosis, (ii) after two cycles of chemotherapy, (iii) at the End of Treatment (EOT), (iv) every three months or every four-six week, depending on whether bone marrow or peripheral blood samples are used, during the two years follow-up period after EOT and (v) before starting the conditioning regimen, if patient is undergoing allo-SCT[72]. Regarding the starting material, the choice between bone marrow and peripheral blood represents an important issue today, especially when considering the goal of reducing patient impact. Generally, as reported in literature, MRD levels in peripheral blood are lower than in bone marrow[99].

Lastly, the choice of the correct marker for monitoring is crucial. Mutations found in *ANKRD26*, *CEBPA*, *DDX41*, *ETV6*, *GATA2*, *RUNX1*, and *TP53* with a higher value of VAF are also excluded, as they are considered germline and noninformative[72]. Additionally, in the last years, growing evidence suggest that some mutations can persist without necessarily representing residual disease, as in the case of DTA mutations, which are excluded due to their association whit age-related clonal hematopoiesis.

3 – Hematopoiesis and Clonal Hematopoiesis

Hematopoiesis (*Figure 9*) is the process by which blood cells - platelet, white blood cells and red blood cells – are formed from pluripotent Hematopoietic Stem Cells (HSCs). These cells possess the ability to self-renewal and sustain the entire immune system throughout life[100].

As hematopoiesis progresses, HSCs differentiate into Multipotent Progenitors (MPPx), which retain multipotency but have lost capacity for self-renew[101,102].

Hematopoiesis can be divided into myelopoiesis and lymphopoiesis. Both HSCs and MPPs express transcription factors from multiple lineages[103]. Myelopoiesis includes processes such as erythropoiesis and megakaryocytopoiesis, while lymphopoiesis leads to the formation of B and T cells.

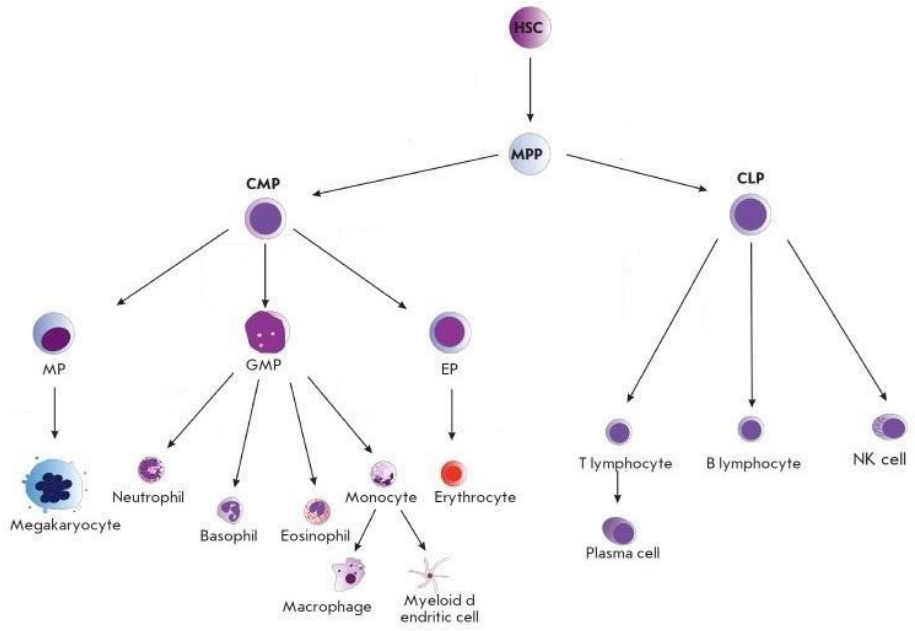

Figure 9: The normal process of hematopoiesis. HSC: Hematopoietic Stem Cell; MPP: Multipotent Progenitor; CMP: Common Myeloid Progenitor; CLP: Common Lymphoid Progenitor; MP: Megakaryocyte Progenitor; GMP: macrophage granulocytic progenitor; EP: Erythropoietin Progenitor. From Vagapova E.R. et al.[104].

DNA damage, along with a failure to repair the damage itself, can lead to mutations, a phenomenon referred to as Clonal Hematopoiesis (CH). Briefly, CH describe the presence of somatic mutations in bone marrow and/or in peripheral blood, resulting from the clonal expansion of hematopoietic stem and progenitor cells[105].

Somatic mutations are part of the body's aging process, but most of these mutations do not appear to have a significant impact[106].

Jaiswal S. et al.[107] reported that the frequency of mutations increases with age: they are rare in individuals under 40, but gradually become more common with age. Additionally, this study highlights that somatic mutations are frequently observed in the general population, with 10% of individuals aged ≥ 70 years presenting these mutations, compared to only 1% of those under 50[108]. The figure below (*Figure 10)* reported the prevalence of somatic mutations in relation to age[109].

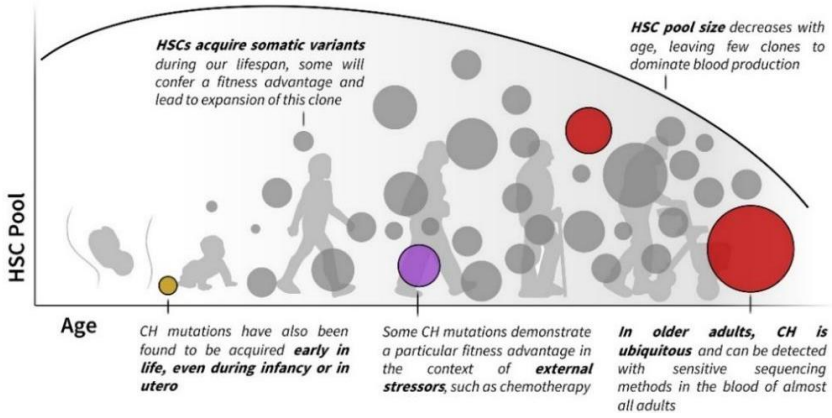

Figure 10: Clonal Hematopoiesis: age-related mutations acquired. Image from "Clonal hematopoiesis: from mechanisms to clinical intervention"[110]. Briefly, somatic mutations are very rare in young individuals, may occur at low frequency in middled age individuals, and become more prevalent in older adults. In older subjects, CH and its subtype, such as CHIP (Clonal Hematopoiesis of Indeterminate Potential) may be present and contribute to the development of hematological malignancies.

In this setting of subjects, the most common mutations detected are *ASXL1*, *DNMT3A* and *TET2*[108,109,111] followed by mutations in *JAK2*, *TP53*, *SF3B1*, *SRSF2* and *CBL*.

Another subset of CH, based on clinical features of the patients, includes: (i) Clonal Cytopenia of Undetermined Significance (CCUS), (ii) Idiopathic Cytopenia of Undetermined Significance (ICUS) and (iii) Clonal Monocytosis of Undetermined Significance (CMUS) and (iv) Clonal Hematopoiesis of Indeterminate Significance (CHIP). The first subset, Clonal Cytopenia of Undetermined Significance, is represented by the

presence of clonal somatic mutation in association with persistence idiopathic cytopenia[1] (≥ 4 months)[112]. The second, Idiopathic Cytopenia of Undetermined Significance, is defined by unexplained cytopenia's when there is no myeloid neoplasm. The third, Clonal Monocytosis of Undetermined Significance, is the presence of persistent monocytosis[2] with somatic mutations in one or more genes associated with myeloid neoplasia. The fourth and most relevant in the context of this project, is Clonal Hematopoiesis of Indeterminate Significance, which is defined by the presence of somatic mutations in genes associated with myeloid neoplasm[113], at Variant Allele Frequency[3] (VAF) of ≥ 0.02%[114], without definitive morphological, histopathological and clinical evidence, though these overt cancer[115] and the risk of developing a malignant tumor is estimated at 0.5-1% annually[116].

The most mutated genes in CHIP are *DNMT3A, TET2, ASXL1, JAK2, PPM1D, TP53, SF3B1* and *SRSF2*[107,109,113,117,118]. In particular, *DNMT3A* is the most commonly mutated gene, with 50-60% of all subjects exhibiting CHIP having a mutation in it[107,108].

CHIP is age-related, much like clonal hematopoiesis. In a recent work, Heuser M. et al.[117], compared three different studies. While there are some differences, there is a clear association between CHIP and age, with almost no events observed in individuals under 40, and the frequency increasing with age.

Clonal Hematopoiesis of Indeterminate Significance is strongly associated with potential progression to AML or MDS[107] with a 3-5 times higher risk of developing AML compared to people without CHIP[116]. However, not all individuals with CHIP have the same risk of developing a hematological malignancy, and it is important to discriminate between transient mutations and driver mutations, that contribute to the development of AML. CHIP has been observed in approximately 70% of patients who later developed AML[119].

The following factors contribute to an increased risk of developing a hematological malignancy: (i) an increase in clone size, resulting to arise in VAF, at least up to a value of 2%[116], (ii) acquisition of additional somatic mutations and (iii) the presence of concomitant

[1] Cytopenia is defined by (i) Absolute Neutrophil Count (ANC) less than $1.8*10^9$/L, (ii) platelet count less than $150*10^9$/L and (iii) hemoglobin level less than 13 g/dL and 12 g/dL in male and female subject, respectively[59.]

[2] Persistent monocytosis is defined as an absolute monocyte count ≥ $0.5*10^9$/L (or ≥10%) relative to the total WBC count.

[3] VAF is the percentage, calculated based on the NGS analysis report, of mutated DNA molecules compared to the total DNA introduced.

somatic mutations in specific genes. The last two factors contribute to what is known as a "clonal complexity", which serves as a strong predictive factor. Four or more mutations at baselines were only observed in patients who went on to develop AML[119].

In particular, the genes commonly involved in the progression to AML are *IDH1*, *IDH2*, spliceosome gene mutations and *TP53*[116,120,121]. Therefore, we can conclude that the risk of developing AML is mutation specific. Additionally, the morphological features of dysplasia tend to be increased[116].

These three factors mentioned above contribute to the evolution from CHIP to the transformation into hematological malignancies (*Figure 11*).

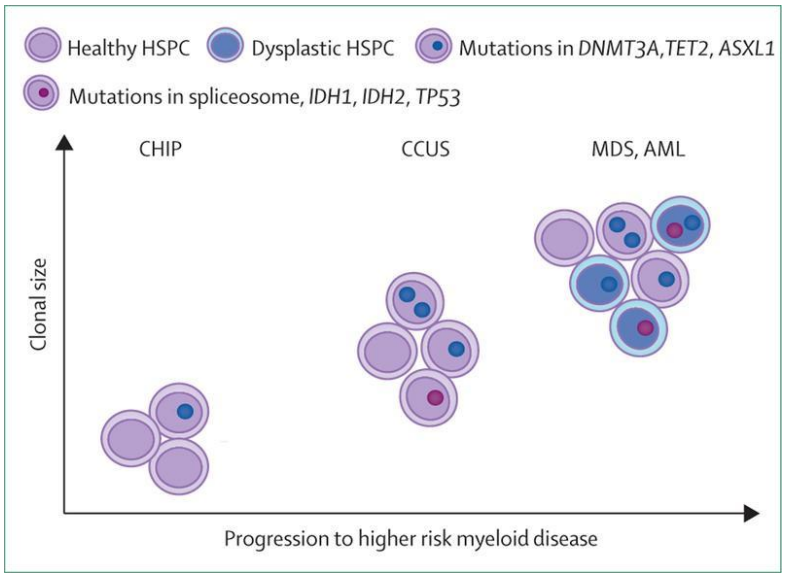

Figure 11: CHIP and risk of progressing to myeloid disease (shown on X-axis). AS mentioned above, certain factors that contribute to malignant transformation; (i) clonal size (and VAF), shown on the y-axis (ii) acquisition of additional somatic mutations, (iii) concomitant somatic mutations in specific genes (IDH1, IDH2, spliceosome genes mutations and TP53) and (iv) increase in morphological features of dysplasia. HPSC: hematopoietic stem and progenitor cell; CHIP: Clonal Hematopoiesis of Indeterminate Significance; CCUS: Clonal Cytopenia of Undetermined Significance; MDS: Myelodysplastic Syndrome; AML: Acute Myeloid Leukemia; From Gondek L.P. et al[116].

Building on the concept of hematopoiesis under physiological conditions (*Figure 9*) and the discussion so far, the concept of "malignant hematopoiesis" leading to the subsequent evolution to AML can be outlined and illustrated.

The progression to AML is straightforward: HSCs and MPPs acquire mutations becoming Leukemic Stem Cell (LSC), denoted as MUT1.a and MUT1.b, respectively. LSC derived from HSCs maintains the capacity for unlimited self-renewal, while LSC from MPPs regain

the capacity for self-renewal. As a result, further mutations can occur in LSC (MUT2 and MUT3). The resulting cells lose the ability to self-renew and cannot differentiate, ultimately becoming blasts cells.

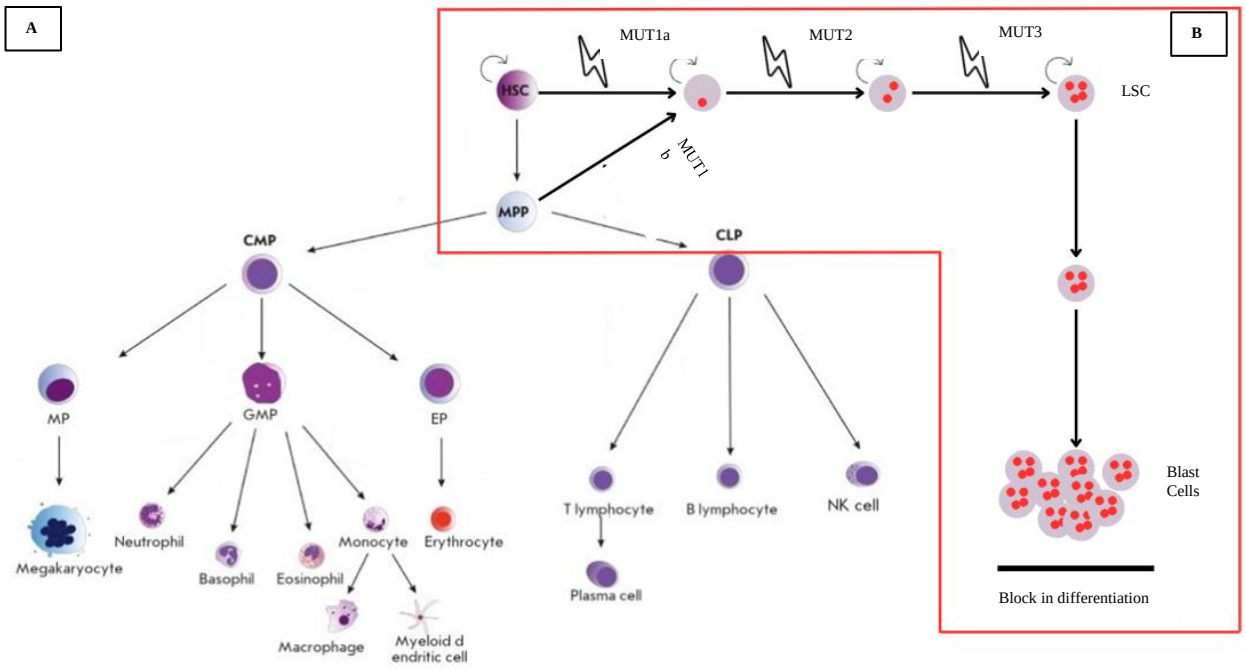

Figure 12: Comparison between normal hematopoiesis (A) and malignant hematopoiesis (B), highlighted in the red box. Two possible scenarios are presented: in the first one, HSC acquired mutations (MUT1a; MUT2; MUT3) and subsequently becomes LSC with unlimited self-reneval (indicated by circle arrow); in the second one a MPP cells acquires a mutation (MUT1b) which restores the cell's capacity for self-renewal and then, as has been seen in HSC, acquires further mutations (MUT2; MUT3). The resulting Leukemia Stem Cell gives rise to blast cells. These have lost their capacity for self-renewal, and will not produce functional cells, despite the mimicry of normal hematopoiesis. HSC: Hematopoietic Stem Cell; MPP: Multipotent Progenitor; CMP: Common Myeloid Progenitor; CLP: Common Lymphoid Progenitor; MP: Megakaryocyte Progenitor; GMP: macrophage granulocytic progenitor; EP: Erythropoietin Progenitor; LSC: Leukemia Stem Cell. Adapted from Vagapova E.R. et al.77.

4 – Aims and objectives

The main objective of this study is to evaluate the feasibility of dPCR to detect and quantify specific gene mutations identified through NGS of AML patients (dPCR-NGS driven platform or methodology).

According to the literature, it is expected that more than 60-70% of patients will present at least one leukemic mutation that can be longitudinally monitored using commercially available platforms.

Considering the primary aim, the approach will be considered feasible if more than 70% of patients have mutations that can be successfully monitored using dPCR.

The secondary aims are:

- To define a threshold for dPCR positivity in the context of MRD evaluation.
- To settle a dPCR tool based on the most recurrent NGS-identified mutational profiles observed in patients with AML and HR-MDS.
- To systematically and longitudinally assess the dPCR NGS-driven profile at multiple clinical time points: at diagnosis, after induction/consolidation therapy, at the end of treatment and, in patients undergoing hematopoietic stem cell transplantation, both before and after that procedure, in order to better quantify the burden of measurable residual disease.

5 – Materials and methods

5.1 – Study design and experimental workflow

The study was designed as an exploratory investigation to evaluate the feasibility of NGS-driven approach for MRD monitoring in bone marrow and peripheral blood.

Patients enrolled in this experimental trial provided written Informed Consent (IC) prior to each procedure. The study Protocol was approved by the Brescia Ethics Committee (Local Reference number: NP4344), on November 5, 2020, under the title: *"Feasibility Study on detection and monitoring of gene-mutations by digital (dPCR) NGS driven in patients with Acute Myeloid Leukemias (AMLs) and High-Risk Myelodysplastic Syndromes (HR-MDSs)-dPCR-NGS Driven*".

Patients' identification and enrollment were conducted at the ASST Spedali Civili di Brescia, specifically within the Hematology and Adult Bone Marrow Transplantation Unit.

The inclusion and exclusion criteria for the study were as follows: (i) subjects ≥ 18 years of age, (ii) patients with new AML or HR-MDS diagnosis and who are candidates for induction and consolidation therapy, with or without allogenic Stem Cells Transplantation and (iii) written informed consent.

The Study involved the collection of PB and BM in newly diagnosed AML or HR-MDS patients, at the following time points: (i) diagnosis, (ii) after induction/ consolidation, (iii) end of treatment, (iv) before and after (30 and 90 days) stem cells transplantation, if applicable, and (vi) anytime in case of relapse.

The study followed AML patients across multiple predefined timepoints, as described below. At each stage, biological and clinical assessments were performed to monitor disease status, progression, response, mutation burden, and MRD. The study design tracked patients through several critical time points, outlined in the flow chart below (*Figure 13*).

- Diagnosis (T0): at this initial timepoint, peripheral blood and bone marrow samples were collected prior to initiation of chemotherapy. Next Generation Sequencing was performed to identify key mutations. Once the mutations of interest were detected and specific probes for dPCR were designed, the dPCR analysis was carried out to determinate the baseline burden mutation, which served as a reference for subsequent longitudinal monitoring.
- Post induction chemotherapy (T1): approximately 28 days after induction therapy, upon hematologic recovery, PB and BM samples were collected again to reassess

mutation burden. This time point was only applicable to patients who were non-responsive to treatment.

- Post consolidation (T2): following the first consolidation cycle, which aimed to eradicate any remaining leukemic cells, PB and BM samples were collected.
- End Of Treatment – EOT (T3): at this stage, the entire planned treatment regimen had been completed. The persistence of MRD at this timepoint indicated the need for close monitoring or additional therapeutic interventions, such as salvage treatment or Hematopoietic Stem Cell Transplantation (HSCT).
- Pre-SCT (T4): for patients eligible for HSCT, pre-transplant samples were collected to assess MRD prior to transplantation.
- Post transplant (T5 and T6): to monitor disease relapses or changes in mutation burden, follow-up samples were collected at 30 (T5) and 90 (T6) days after transplantation. This evaluation was critical for early detection of relapses, allowing timely intervention with additional therapies such as Donor Lymphocyte Infusions (DLI). Close monitoring at these time points provided valuable insight into transplant efficacy and the likelihood of long-term remission.
- Anytime, in case of relapse: in addition to the scheduled time points, patients were monitored at any time in the event of evidence of relapse.

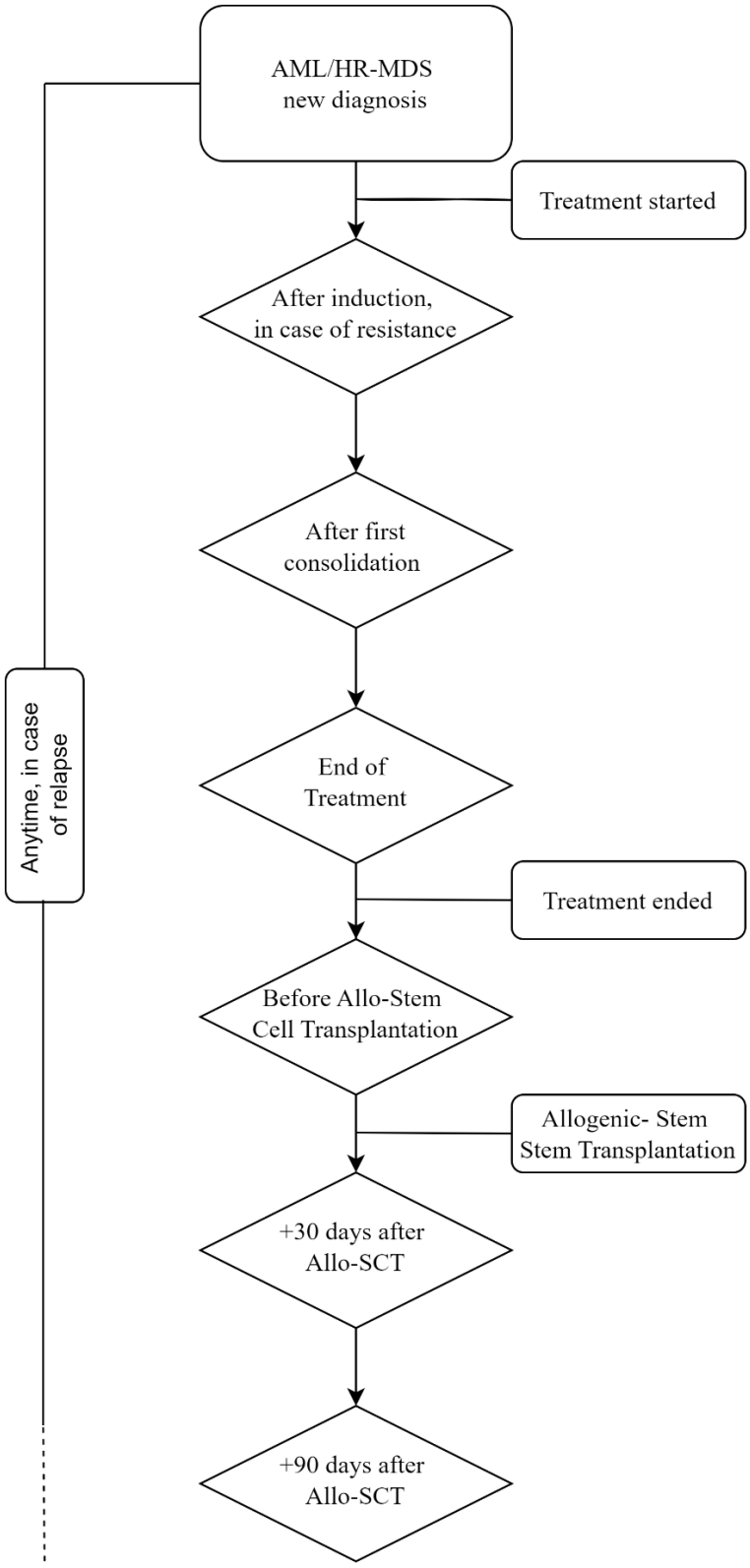

Figure 13: Study flowchart.

5.2 – Patients

This trial was conducted in patients with newly diagnosed AML or HR-MDS at Hematology Units of ASST Spedali Civili di Brescia.

The local research and ethics committee approved the study protocol (NP4344), and all patients provided written informed consent. The trial adhered to the principles of the Declaration of Helsinki.

A total of 32 patients with new AML diagnoses were included in the study.

The evaluation is currently ongoing and now we present the preliminary results concerning the first 14 patients.

5.3 – Patients' monitoring

To accurately define the pathology following diagnostic suspicion, a series of routine tests are prescribed by physicians, according to local practice and national/internation guidelines. These tests are conducted at the "*Laboratorio Centrale di Analisi Chimico-Cliniche*" of ASST Spedali Civili di Brescia. The tests include the analysis of leukemia-associated immunophenotype, mutations in *FLT3-ITD* and *FLT3-TKD*, *NPM1* and the overexpression of *WT1*. If one or more of these markers are detected, they are used as leukemia-specific markers for routine monitoring of MRD. The frequency and timing of MRD monitoring follow international guidelines and are adapted as necessary to clinical practice.

LAIP is detected and monitored through flow cytometry, with results reported as a percentage of leukemic cells. The positivity cutoff for LAIP is set at $3.5*10^{-4}$ (0.035), as defined by Buccisano and colleagues[122]. Mutations in *FLT3*, including both ITD[123] and TKD[124], *NPM1* and *WT1* overexpression are assessed using real-time PCR. Results for *FLT3-ITD*, *FLT3-TKD*, and *NPM1* are reported as either present or absent. In contrast, *WT1* overexpression is quantified and expressed as a percentage. Values above a specific threshold indicate overexpression. For *WT1* two different positivity cutoffs are set, depending on whether the bone marrow or the peripheral blood is being evaluated. These cutoffs are set at 250 e 50 number of copies/10^4 ABL copies, respectively[125]. In some cases, NPM1 mutations are not only reported as present or absent but also quantified and they are expressed as NPM1A/ABL*10^4.

5.4 – Samples processing

As previously reported, peripheral blood and bone marrow blood samples were collected in tubes containing anticoagulants, such as K3EDTA. Leukocytes and plasma were isolated from the peripheral blood, while mononuclear cells were isolated from the bone marrow after collecting both blood samples. For bone marrow blood samples, it was estimated that approximately 10 mL of blood was required as the starting material to obtain at least 150-200 ng of DNA. For peripheral blood, approximately 15 mL of whole blood was needed as starting material to obtain an adequate number of cells. In addition, both peripheral blood and bone marrow blood were processed within two hours of collection to ensure optimal quality and quantity of the extracted material.

5.4.1 – Mononuclear cells isolation

Bone marrow samples were collected via needle aspiration to isolate mononuclear cells using the density gradient separation method described by Böyum A.[126]. The resulting buffy coat was subjected to two sequential washes or, when required, erythrocyte lysis to obtain a purified cell pellet. The pellet was either used immediately for downstream applications or stored at -80°C for future analyses.

5.4.2 – Plasma and leucocyte cells isolation

Peripheral blood samples, collected according to clinical practice, were processed to isolate leukocytes following a standardized protocol. After centrifugation at 2,800 rpm for 15 minutes, plasma was aliquoted and stored at -20°C. Than the leucocyte layer was collected and subjected initially to two lysis steps and subsequently to two washes with PBS. The pellet thus obtained, if not used immediately, is stored at -80°C.

5.5 – DNA isolation

DNA extraction was performed using the PureLink™ Genomic DNA Mini Kit (Invitrogen, ThermoFisher Scientific, Cat. No. K182002, Waltham, MA, USA), following the manufacturer's protocol which includes two main steps: (i) lysis and (ii) purification. The DNA obtained was subjected to quantitative (concentration expressed as ng/uL) and

qualitative (260/280 nm and 260/230 nm ratios) control via NanoDrop 2000c spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA) and Qubit™ High Sensitivity (HS) Fluorometer (ThermoFisher Scientific Waltham, MA, USA), and subsequently stored at -20°C until it was used.

5.6 – NGS analysis

After DNA extraction, NGS analysis was performed either for clinical routine or research purposes. The entire workflow followed a CE-IVD certified method. Targeted sequencing of myeloid-associated mutations was performed using the SOPHiA DDM™ Dx Myeloid Solution (Panel ID MYS_v1), (SOPHiA GENETICS, Saint-Sulpice, Switzerland) according to the manufacturer's instructions. The MYS panel covers the coding regions, sequence flanking regions (± 25bp) and ITDs of 30 genes associated with Myelodysplastic Syndromes, Myeloproliferative Neoplasms and Leukemia.

NGS analysis is based on three fundamental steps: (i) library preparation, (ii) sequencing and (iii) bioinformatics analysis.

Library preparation consists of several steps: (i) preparation, (ii) enzymatic fragmentation, end repair and A-tailing, (iii) ligation, (iv) post ligation clean up, (v) dual size selection, (vi) library amplification, (vii) post amplification clean up, (viii) individual library quantification and quality control, (ix) library pooling, (x) hybridization (xi) streptavidin beads preparation, (xii) binding of hybridized targets to the beads, (xiii) wash streptavidin beads to remove unbound DNA, (xiv) post-capture amplification, and (xv) post capture amplification cleans up. Once the libraries are ready, we proceed with sequencing.

Sequencing was performed on two different platforms: when sequencing was ordered for diagnostic purposes, Illumina's MiniSeq sequencer was used with a minimum read depth of 5000x and a detection limit of 3%, while when sequencing was ordered for research purposes, Illumina's MiSeq sequencer was used with a 600-cycle V3 flow cell with a minimum read depth of 1000x and a detection limit of 1%. The final step is bioinformatic analysis, starting from the .FASTQ file generated by the sequencer. The analysis is performed using SOPHiA DDM™ software, which enables the identification of all mutations in the genes includes in the MYS gene panel. SOPHiA DDM™ software includes advanced algorithms for read alignment, SNP and small indel detection (PEPPER), CNV detection (MUSKAT) and functional variant annotation (MOKA).

5.7 – Marker selection and design

Following the detection of mutations at diagnosis, those classified as AML-related, likely AML-related or potentially AML-related[9] were selected for subsequent MRD monitoring using dPCR.

Reference sequences were retrieved using databases such as Ensembl and ClinVar, based on variant information provided by the SOPHiA DDM™ software, and were used to design patient-specific probes. These probes were designed using bioinformatics tools and are of the TaqMan® hydrolysis probe type, consisting of an oligonucleotide probe complementary to the target sequence, labelled with a fluorophore at one end and a quencher at the opposite end. Four fluorophores were used for multiplexing (i) VIC, (ii) FAM, (iii) ABY and (iv) CY5. The average probe length was set at approximately 400 nucleotides, with the mutation site positioned centrally. The probes were designed to have a balanced Guanine (G) and Cytosine (C) content, ranging approximately from 40 to 60%.

A total of 18 probes were designed, incorporating two different markings combinations, and were used in multiplex dPCR analyses. Some assays are labelled with VIC/FAM, where the mutant allele was detected in the FAM channel and the wild-type alleles in the VIC channel. Other assays marked in ABY/CY5 combination, in which copies of the mutant allele are positive in the CY5™ channel, while copies of the wild-type alleles are positive in the ABY™ channel.

5.8 – dPCR analysis

Digital PCR analysis was conducted using the QuantStudio™ Absolute Q™ Digital PCR System (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's standardized protocol. All four fluorescence detection channels (VIC, FAM, ABY, CY5) were utilized to enable and optimize the multiplexing strategy. Data acquisition and analysis were subsequently conducted using QuantStudio™ 3D AnalysisSuite Cloud Software (Thermo Fisher Scientific, Waltham, MA, USA). In the figure below (*Figure 14*) the complete workflow of the dPCR is reported.

Figure 14: dPCR workflow used to monitor MRD in AML patients over multiple time points. Peripheral blood, bone marrow and exosomes were analyzed to detect and quantify gene mutations with high sensitivity and accuracy.

5.9 – Statistical analysis

Statistical analyses were performed using RStudio software (version 4.3.2). Descriptive statistics were used to summarize patient demographics and mutation frequencies derived from the NGS analysis.

The sensitivity and specificity of dPCR for Measurable Residual Disease detection were compared with traditional methods, including RT-PCR and immunophenotyping, to assess the diagnostic performance of dPCR approach.

6 – Results

- **Patients' characteristics**

To date, 32 patients have been enrolled in the study. Among the 32 patients, 14 (44%) have already been analyzed for all the scheduled time points using the dPCR NGS-driven approach (*Figure 15*). Conversely, 18 patients are currently pending analysis.

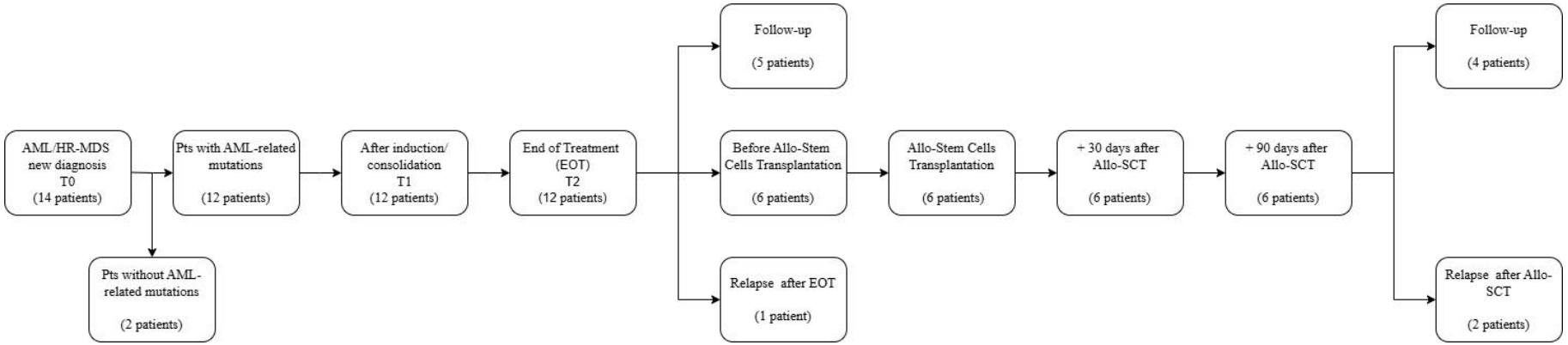

Figure 15: Flowchart illustrates the enrolled and analyzed patients and their progression through the study. The chart highlights the number of patients at each timepoint.

Twelve of fourteen patients (86%) had at least one AML-related mutation that could be followed by dPCR across all the timepoints. Considering the 12 patients analyzed, 6 (50%) underwent hematopoietic stem cell transplantation, while the other 6 starting the follow up without being allotransplanted after EOT. Overall, 3 patients (25%) have experienced disease relapse, with a mean time from diagnosis of 442 days (range: 344 – 539). Among the latter, 2 patients (67%) relapsed after allo-HSCT, while the remaining one (33%) after the end of treatment (T2).

The median duration of follow-up was 305 days (IQR 394 - 539 days).

Table 7 summarizes the main clinical and hematological characteristics of the patients at diagnosis.

Variable	Total patients analyzed n=12
Mean age (y), (range, (y))	58 (53 – 70)
Sex	
Male, n° (%)	6 (50%)
Female, n° (%)	6 (50%)
Diagnosis (according to WHO 2022)	
AML with defining genetic abn, n° (%)	10 (83%)
AML, myelodysplasia-related, n° (%)	2 (17%)
Karyotype	
Normal, n° (%)	10 (83%)
Abnormal, n° (%)	2 (17%)
Complex, n° (%)	1 (8%)
Molecular Biology (RT-qPCR, Nested-PCR or HPLC)	
NPM1, n° (%)	7 (58%)
FLT3- ITD, n° (%)	3 (25%)
FLT3-TKD, n° (%)	1 (8%)
WT1, n° (%)	10 (83%)
ELN risk stratification (according to ELN guidelines 2022)	
Favorable, n° (%)	4 (≈33%)
Intermediate, n° (%)	4 (≈33%)
Adverse, n° (%)	4 (≈33%)

Hb, g/dL Mean (range)	10.55 (8.60 – 14.30)
WBC, $x10^6$/L Median (interquartile)	7.35 (2.15 – 18.16)
Platelet, $x10^9$/L Mean (range)	115.5 (14.0 – 202.0)
Bone marrow blasts, mean % (range)	64.7 (24.0 – 95.0)
Circulating blasts, mean % (range)	45.91 (5.0 – 92.0)

Table 7: Baseline clinical-hematological characteristics. Y: years. Abn: abnormalities. Hb: hemoglobin. WBC: White Blood Cells.

According to the fifth edition of the WHO classification[55], the main subtype of AML was Acute Myeloid Leukemia with defining genetic abnormalities (10/12, 83%), followed by Acute Myeloid Leukemia Myelodysplasia-related (2/12, 17%).

Risk stratification according to the ELN 2022 guidelines[56] identified four out of twelve patients (≈33%) as favorable risk, four (≈33%) as intermediate risk and four (≈33%) as adverse risk. Among these, two patients (17%) exhibited cytogenetic abnormalities, one of which was a complex karyotype

In *Figure 16*, the types of markers of MRD detected at diagnosis were reported.

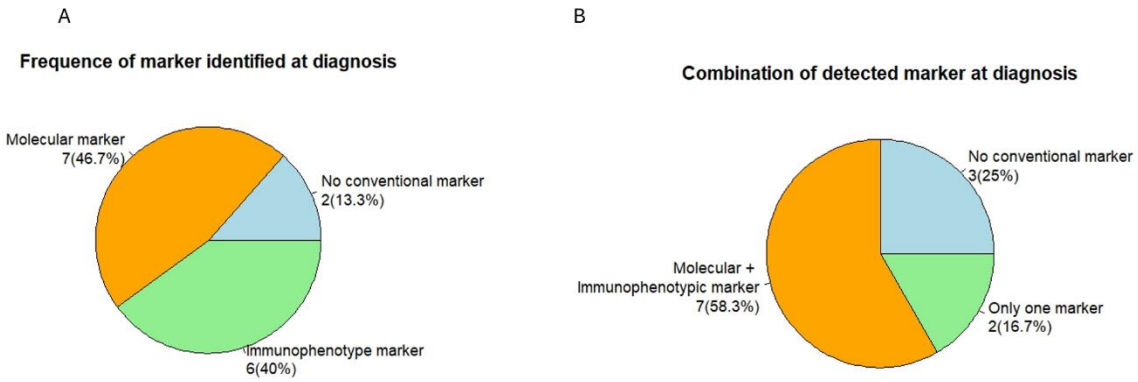

Figure 16: Box A represents the frequency of markers detected at diagnosis. Among the patients analyzed, molecular markers were the most frequent (n = 7), followed by immunophenotypic and cytogenetic markers (n = 6 and 2, respectively). Each category represents the presence of at least one marker belonging to that class, regardless of any co-occurrence with other types. Box B represents the distribution of combinations of molecular, immunophenotypic, and cytogenetic markers detected at diagnosis. Most patients had only one type of marker (n = 5).

- **NGS analysis at diagnosis**

NGS identified at least one leukemia-associated mutation suitable for monitoring via dPCR

in 12 out of 14 patients (86%). Overall, 15 different genes were mutated in the analyzed patients, with a median of 3 mutations per patient. The most frequently mutated gene was *NPM1*, found in 7 out of 12 (58%) analyzed patients (*Figure 17*). Multiple mutations within the same gene were observed in several patients, resulting in a total of 31 total unique mutational events; *DNMT3A* exhibited the highest number of mutations, with a total of 6 distinct mutational events (*Figure 18*). Table 8 displays the mutations identified in individual patients.

Id patient	**Gene**	**Variant/protein**	**VAF (%)**
NGS-dPCR001	No mutations detected by NGS analysis at diagnosis		
NGS-dPCR002	*ASXL1*	c.1934dupG/p.(Gly646Trpfs*12)	36.1
	IDH2	c.419G>A/p.(Arg140Gln)	47.5
	SRSF2	c.284C>A/p.(Pro95His)	43.5
	RUNX1	c.941_942del/p.(Ser314Cysfs*285)	3.5
	RUNX1	c.1048_1049insG/p.(Pro350Argfs*250)	23.3
	RUNX1	c.1075_1078dup/p.(Val360Alafs*241)	10.8
NGS-dPCR003	*IDH1*	c.394C>T/p.(Arg132Cys)	43.4
	NPM1	c.860_863dup/p.(Trp288Cysfs*12)	39.5
	ZRSR2	c.1338_1343dup/p.(Ser447_Arg448dup)	90.1
NGS-dPCR004	*DNMT3A*	c.2644C>T/p.(Arg882Cys)	44.3
	FLT3	c.2503G>T/p.(Asp835Tyr)	5
	FLT3	c.2508_2510delCAT/p.(Ile836del)	2.8
	NPM1	c.860_863dupTCTG/p.(Trp288Cysfs*12)	37.2
	NRAS	c.35G>A/p.(Gly12Asp)	1.4
	NRAS	c.35G>C/p.(Gly12Ala)	30.3
	NRAS	c.37G>C/p.(Gly13Arg)	1.5
NGS-dPCR005	*DNMT3A*	c.958C>T/p.(Arg320*)	45
	IDH2	c.419G>A/p.(Arg140Gln)	46
	NPM1	c.860_863dupTCTG/p.(Trp288Cysfs*12)	38
NGS-dPCR006	*FLT3*	c.1715A>G/p.(Tyr572Cys)	3
	RUNX1	c.319C>T/p.(Arg107Cys)	41.3
	WT1	c.1283G>A/p.(Cys428Tyr)	18.2
	WT1	c.1390G>A/p.(Asp464Asn)	2.9
NGS-dPCR007	*CEBPA*	c.111_124del/p.(Gly38Serfs*65)	36.1
	CEBPA	c.918_938delinsGAG/p.(Asn307_Lys313delinsArg)	35.5
NGS-dPCR008	*DNMT3A*	c.2323-2A>G/p.(?)	42.4
	IDH2	c.419G>A/p.(Arg140Gln)	40.7
	NPM1	c.860_863dupTCTG/p.(Trp288Cysfs*12)	33.7
NGS-dPCR009	*ASXL1*	c.3543C>G/p.(Ser1181Arg)	49.6
	NPM1	c.860_863dupTCTG/p.(Trp288Cysfs*12)	34.1

	TET2	c.3788G>A/p.(Cys1263Tyr)	42.8
	TET2	c.3860_3861del/p.(Phe1287Trpfs*12)	41.5
NGS-dPCR010	No mutations detected by NGS analysis at diagnosis		
NGS-dPCR011	*ASXL1*	c.1934dupG/p.(Gly646Trpfs*12)	28.5
	IDH2	c.419G>A/p.(Arg140Gln)	33.4
	U2AF1	c.467G>A/p.(Arg156His)	6.4
NGS-dPCR012	*DNMT3A*	c.2185C>T/p.(Arg729Trp)	47.2
	DNMT3A	c.2510C>G/p.(Ser837*)	44.8
	ETV6	c.50A>G/p.(Tyr17Cys)	50.8
	IDH2	c.419G>A/p.(Arg140Gln)	46.2
NGS-dPCR013	*IDH1*	c.394C>T/p.(Arg132Cys)	9.1
	NPM1	c.860_863dupTCTG/p.(Trp288Cysfs*12)	10.1
NGS-dPCR014	*DNMT3A*	c.2645G>A/p.(Arg882His)	46.2
	NPM1	c.860_863dupTCTG/p.(Trp288Cysfs*12)	45

Table 8: Table summarizing the molecular characteristics of the patients. For each patient, the mutated gene, the specific variant and protein consequence are reported. ID pt: Patient Identification number.

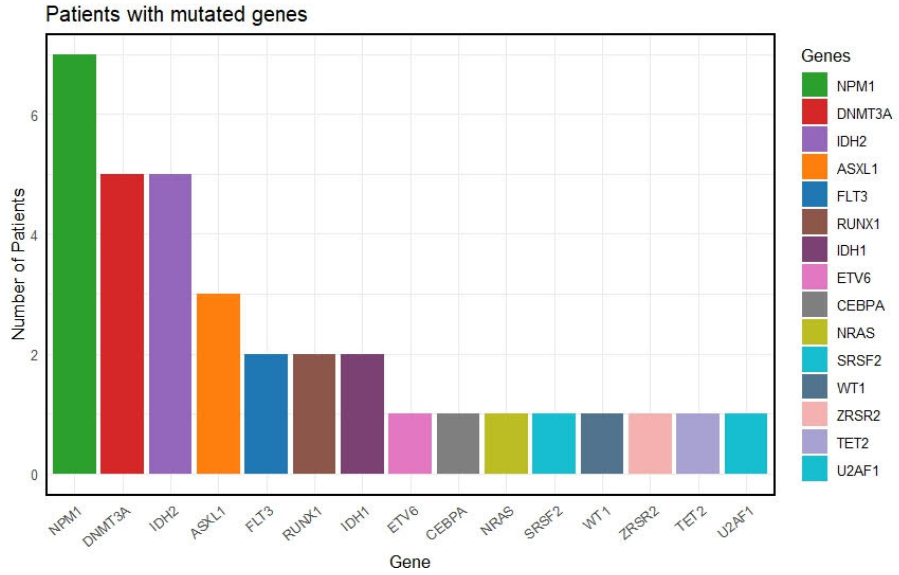

Figure 17: Bar-plot illustrating the distribution of gene mutations identified in AML patients at diagnosis through NGS analysis. Each bar represents the number of patients with mutations in a specific gene.

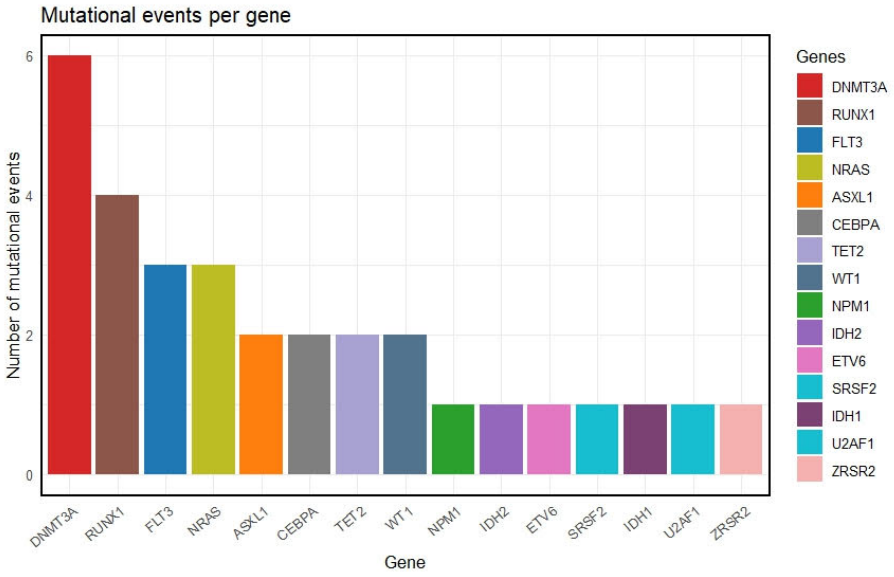

Figure 18: Bar-plot illustrating all mutational events in genes identified in AML patients at diagnosis by NGS analysis.

- **Feasibility of the study**

Twelve out of fourteen patients (86%) had at least one mutation at diagnosis by NGS, which are known to be related or potentially related to AML[9], and, therefore, suitable for dPCR monitoring (*Figure 19A*). Conversely, 2 patients did not harbor any mutations detected by NGS. All the mutations identified at diagnosis by NGS were successfully detected by dPCR in bone marrow and peripheral blood samples, using both commercial or custom probes (*Figure 19B*).

These two data highlight the feasibility of an NGS-driven dPCR approach for studying MRD in AML patients.

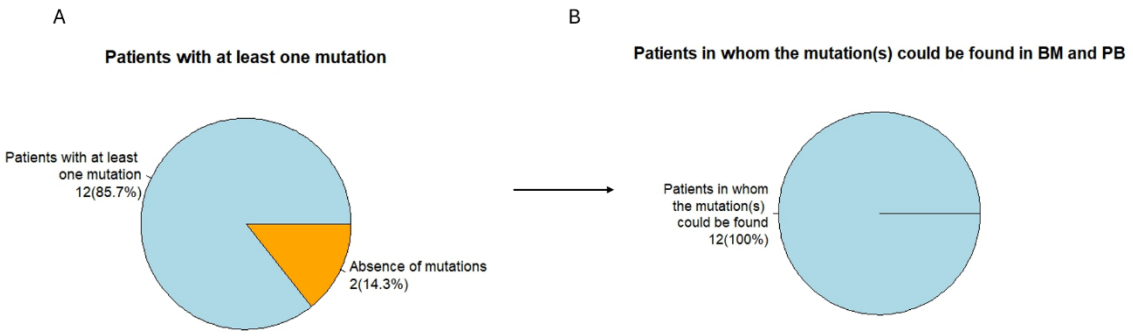

Figure 19: Proportion of patients with at least one mutation identified via NGS and suitable for dPCR monitoring (A), and detectability of target mutations in both BM and PB (B); of note, all selected mutations were simultaneously detected in BM and PB.

- **dPCR analysis**

Regarding dPCR analysis, probe design was guided by the NGS results obtained at diagnosis. Custom probes were designed for the majority of the mutations, while commercially available probes were used when possible (*Figure 20*). As noted, custom assays comprised 78% of all probes, while the remaining 22% sourced commercially.

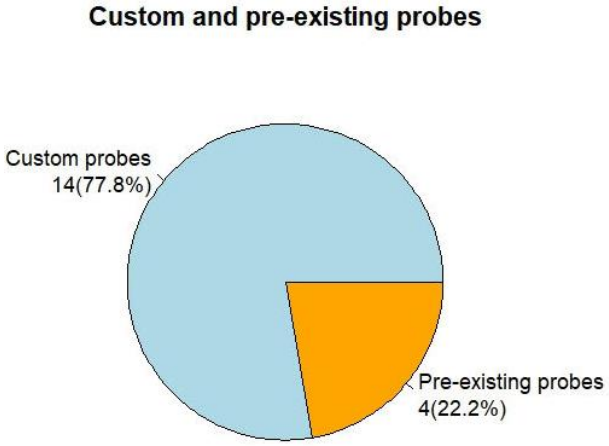

Figure 20: Custom versus commercial probes.

Furthermore, the concurrent detection of the wildtype and mutated sequence of the same gene worked as internal control for the dPCR assays.

- **dPCR NGS-driven approach for studying MRD**

The NGS-driven dPCR approach for MRD monitoring can be used in two different scenarios.

Notable 3/12 (25%) showed MRD positivity with dPCR preceding relapse. In one (33%) there were no conventional markers of disease. Of the other two relapsed patients with markers detectable also with RT-PCT or MFC, dPCR was more sensitive than traditional methods, showing MRD positivity when both RT-PCR or MFC were negative (please see next section for more details).

1) **MRD monitoring in the absence of conventional markers**

   NGS-driven dPCR allows to monitor MRD in patients who lacked traditional molecular markers or immunophenotypic profiles (LAIP). Specifically, this has been possible in 2 out of 12 patients (17%), predicting relapse in 1 case. For example, patient 6 (*Figure 21*) presented with normal karyotype and without molecular or immunophenotypic markers and, therefore, it was not possible to detect MRD by RT-PCT or MFC. Conversely, the

patient presents mutations in *FLT3, RUNX1* and *WT1* by NGS at diagnosis, which were then followed by dPCR. The patient achieved complete remission after induction, but, unfortunately, relapsed after the end of treatment (T2). Using the NGS-driven dPCR approach, patient-AML related mutations [FLT3 (c.175A>G), RUNX1 (c.319C>T), and WT1 (c.1283G>A; c.1390.G>A)] were successfully monitored by dPCR, and an increase of mutations' burden (MRD positivity) was already detected at the EOT (T2), predicting the relapse of the disease. This example highlighted the potential advantage of this approach in patients lacking markers by RT-PCR of MFC.

In addition, a strong concordance between dPCR results in peripheral blood and bone marrow samples have been observed.

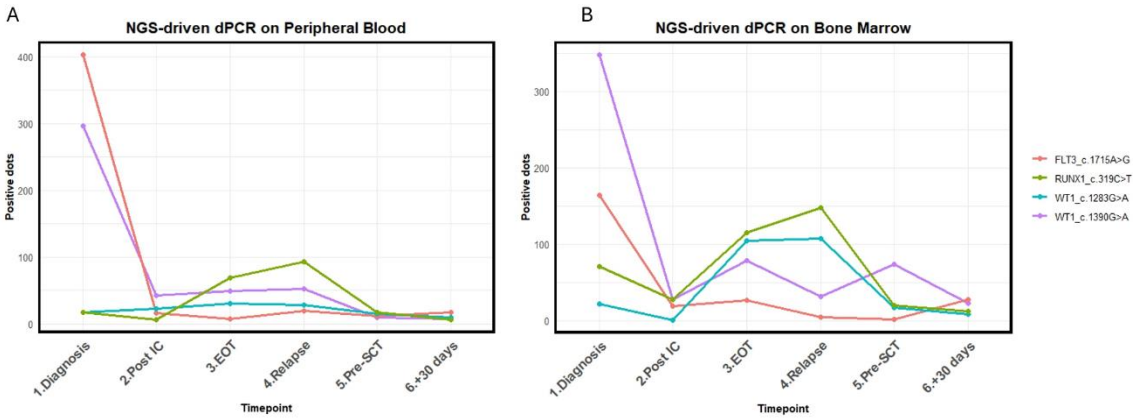

Figure 21: NGS-driven dPCR results in peripheral blood (A) and bone marrow (B) were highly concordant. Moreover, dPCR NGS-driven enabled MRD detection even in patients who lack conventional MRD markers.

2) MRD monitoring in the presence of traditional markers

When conventional MRD markers were present, dPCR showed concordance with conventional methods in 6 patients out of 10 (60%), underscoring the reliability of dPCR as a clinical tool.

In other cases, dPCR demonstrated higher sensitivity (up to 10^{-5}), compared to RT-qPCR or flow cytometry. In 2 out of 10 (20%), dPCR successfully detected low-level residual leukemic cells when the LAIP by MFC or RT-PCR resulted negative. Specifically, patient 002 presented at diagnosis with normal karyotype, WT1 overexpressed, a defined LAIP (CD34+CD117+CD13+CD33dim/-HLADR+) and mutations in *ASXL1*, *IDH2* and *RUNX1* identified through NGS analysis. The patient who was MFC-MRD negative but still dPCR-MRD positive prior to allo-SCT and

underwent transplantation based on MFC negativity. After allo-SCT, MFC remained negative while dPCR initially turned negative but later became positive, predicting clinical relapse (*Figure 22*). Similarly, in another patient with both LAIP and NPM1/FLT3 mutations detected by RT-qPCR at diagnosis, MFC-MRD was negative at the end of treatment (EOT, T2), while NPM1 remained positive by dPCR, showing only a minimal decrease in levels compared to diagnosis (<1 logarithm). Conversely, RT-PCR showed a significant decrease in *NPM1* levels (up to 4 logarithms) at EOT. This patient subsequently underwent allo-HSCT but experienced a molecular relapse six months after the transplant, highlighting the higher sensitivity of dPCR compared to both MFC and RT-qPCR for MRD detection. This last discussed case was described but not reported as a figure.

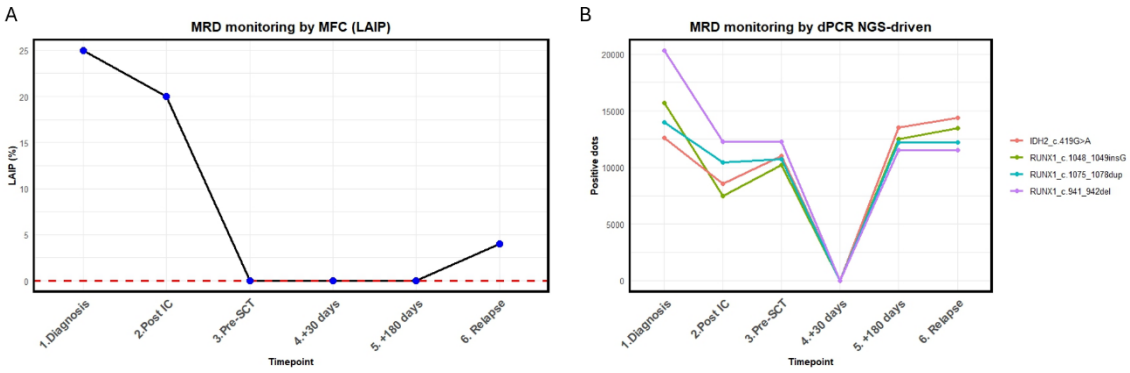

Figure 22: dPCR NGS-driven detected residual leukemic cells when flow cytometry results were negative, providing earlier relapse prediction.

Notably, patient 13, who presented with LAIP and *NPM1* and *FLT3* mutations at diagnosis, achieved MRD negativity by both MFC and RT-qPCR before allo-HSCT and one month after it. Conversely, dPCR demonstrated higher sensitivity, detecting MRD positivity as early as one month after allo-HSCT and predicting molecular relapse, which was confirmed by RT-PCR three months after transplant (*Figure 23*).

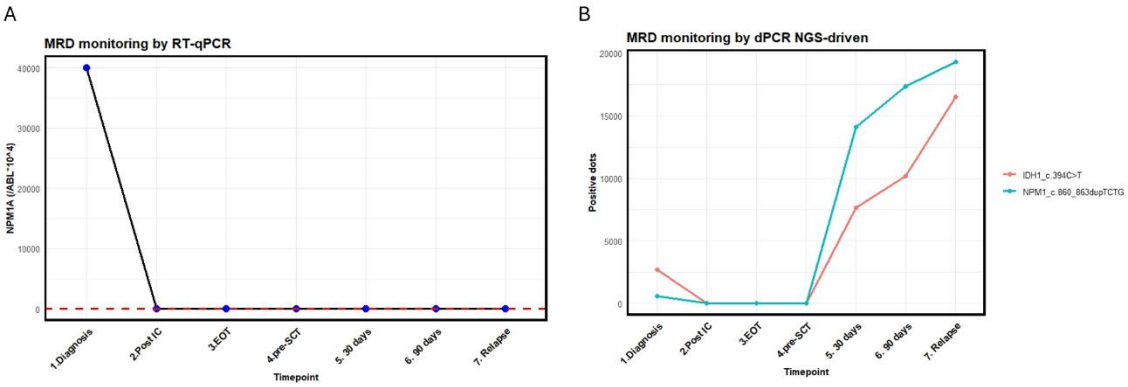

Figure 23: dPCR NGS-driven detected residual leukemic cells when RT-qPCR results were negative (EOT, pre-SCT and 30 days after allo-HSCT), or slightly increased (90 days after allo-HSCT).

Finally, when comparing dPCR with RT-qPCR, we observed a patient who, despite showing low persistence of *NPM1* in bone marrow at the end of treatment by RT-qPCR (with a significant reduction from baseline, -3 logarithms), exhibited a significant increase in *NPM1* dots by dPCR at EOT, reaching levels comparable to those at diagnosis and raising concern for a possible relapse. Indeed, the patient relapsed three months after EOT.

7 – Discussion

Acute Myeloid Leukemia is a heterogeneous hematological neoplasm characterized by the clonal proliferation of immature myeloid cells biologically and genetically heterogenous.
Although most patients achieve initial remission, relapse rates remain high[76]. Monitoring Measurable Residual Disease (MRD) is crucial for detecting residual leukemic cells that can lead to relapse[72]. MRD monitoring has been significantly enhanced by recent technological advances, with several analytical techniques now offering very high sensitivity, even down to 10^{-5} with RT-PCR or MFC. However, traditional MRD monitoring methods have limitations: RT-qPCR tracks only a limited number of molecular markers, and immunophenotyping may miss specific immunophenotypic markers (LAIP). While Next Generation Sequencing (NGS) provides valuable insights into AML's genetic heterogeneity, it lacks the precision needed for accurate mutation quantification[3]. Recently, digital polymerase chain reaction (dPCR) has emerged as a more sensitive and accurate method[4,5] for MRD monitoring, enabling the precise quantification of specific mutations at low levels, providing a detailed and dynamic indication of disease burden and progression both during and after treatment, thus allowing personalized treatment and improving the chances of long-term success.
Unlike qPCR, dPCR offers the advantage of absolute quantification without the need for standard curves, making it a robust tool for detecting specific mutations, even at minimal levels.
Despite the availability of multiple MRD monitoring strategies, there is not a universal approach for detecting residual leukemic cells. To date, MRD monitoring and its use in clinical routine requires further refinement, standardization and harmonization of analytical platforms. Each technique has its limitations: multiparametric flow cytometry is operator-dependent and may be influenced by immunophenotypic shifts over time, potentially reducing accuracy and sensitivity. Similarly, RT-qPCR is limited to specific molecular markers and applicable only to patients with well-characterized molecular aberrations.
To overcome the current limitations of MRD assessment, we aimed to combine the ability of NGS at diagnosis to capture the biological and genetic heterogeneity of the disease with the high sensitivity of dPCR for the longitudinal monitoring of AML-associated mutations. This approach was implemented in the context of the study "*Feasibility Study on detection and monitoring of gene-mutations by digital (dPCR) NGS driven in patients with Acute*

Myeloid Leukemias (AMLs) and High-Risk Myelodysplastic Syndromes (HR-MDSs)- "dPCR-NGS Driven Study" that we are conducting at ASST Spedali Civili di Brescia.

The main objective of this study was to evaluate the feasibility of dPCR for detecting and quantifyng specific gene-mutations identified via NGS in AML patients and subsequently select for MRD monitoring. Secondary objectives include identifying a potential threshold of positivity, establishing a dPCR tool based on the most recurrent NGS-identified mutations in AML patients, and systematically assessing the dPCR-NGS-driven profile at multiple clinical timepoints, comparing it with standard MRD monitoring methods.

Although BM is the classic source for MRD monitoring, PB analysis may offer a less invasive alternative, making it possible to monitor more frequently. Therefore, at each timepoint, we analyzed AML mutations by dPCR in both PB and BM.

At present, 32 patients with newly diagnosed AML were included in the study. The evaluation is currently ongoing and now we present the preliminary results from the first 12 patients.

The NGS-driven dPCR approach proved to be feasible. Indeed, 10 out of 12 (86%) patients had at least one AML-related mutation that could be monitored by dPCR. Additionally, all identified mutations were tracked across the scheduled timepoints. Given that RT-qPCR is applicable to only 45-50% of AML patients due to its reliance on specific molecular markers, we considered our approach feasible if more than 70% of patients carring mutations could be successfully monitored using dPCR. In the first instance, more than 70% of patients had at least one mutation monitorable via dPCR and, in addition, all mutations could be found, both in bone marrow and peripheral blood.

It is important to note that, at this early stage, the design of customized probes was required in the majority of cases. However, this limitation is expected to decrease over time, as the probes developed for the initial patients will become available for broader application in future cases.

Regarding MRD monitoring, our results suggest that dPCR-NGS driven may apply in two distinct scenarios: (i) MRD monitoring in the absence of conventional markers, and (ii) MRD monitoring in the presence of traditional markers.

In the first scenario characterized by a complete absence of standard MRD markers, we leveraged diagnostic NGS data to identify patient-specific mutations, which were subsequently used as custom targets for dPCR monitoring, extending the reach of molecular MRD assessment to a broader subset of patients. In these cases, a personalized approach based on patient-specific mutations allows MRD monitoring to be extended to a wider

population, including those patients who might not otherwise benefit from accurate molecular surveillance. This confirms the usefulness of the dPCR as a complementary tool.
In the second scenario, when at least one marker of disease is present, three different situations can be delineated: (i) concordant results with standard methods, (ii) molecular response preceding conventional molecular clearance, (iii) dPCR serves as an early indicator or predictor of clinical relapse.
A strength that emerged from our analysis was the consistency between the results obtained by dPCR and those derived from the methods used in clinical practice. Indeed, in most cases (60%), dPCR was concordant with standard clinical assessments, providing further validation of this approach. This overlap between technologies, indeed, underscores the reliability of dPCR as a tool that can be integrated into daily clinical practice.
Where, on the other hand, discordance was observed, dPCR provide early detection of relapse, thus offering the potential to predict relapse before it became evident with traditional methods. Notably, in some patients, dPCR detected an increase in mutational earlier than RT-qPCR or MFC analyses, predicting relapse. This suggests that dPCR may act as an early warning system, thanks to its high sensitivity (up to 10^{-5}) and allowing for closer molecular monitoring and possible adoption of pre-emptive therapeutic strategies (e.g., intensification of therapy, donor lymphocyte infusion, et cetera). Finally, dPCR results were not in agreement with MFC results in at least one case. Specifically, before allo-HSCT, the patient was MFC-MRD negative, whereas dPCR detected low levels of leukemic cells. After allo-HSCT, MFC continued to show negativity, while dPCR, despite an initial negative result, turned positive, thus allowing early detection of relapse. This observation underscores the higher sensitivity of dPCR, even when compared with traditional methods, which are typically considered highly sensitive, highlighting its potential for earlier and more reliable MRD monitoring.
From the point of view of molecular clearance, on the other hand, dPCR showed, before standard molecular biology showed an appreciable decrease in transcript, a significant reduction to complete negativity of the target mutation. This suggests how, due to its greater analytical sensitivity and quantitative resolution, dPCR, can detect earlier and more accurately the dynamics of residual disease clearance, providing a "*real-time*" picture of disease status, although this further aspect needs further investigation.
Moreover, the concordance between bone marrow and peripheral blood in detecting and quantifying target mutation with dPCR suggest that PB coulde serve as a valid non-invasive

alternative to bone marrow for MRD monitoring in the near future, without compromising the accuracy of molecular evaluation.

Additionally, the multiplex method that our work offers enables the simultaneous detection of several leukemia clones in a single reaction. This approach implies, first, a reduction in the technical time required to obtain results, as well as a decrease in the potential for error. Moreover, it proves to be cost-effective by requiring fewer reagents and consumables, ultimately contributing to improved patient management.

The next steps will involve validating the results obtained on a larger cohort of patients, defining a positive cutoff (currently not applicable due to the limited number of cases and heterogeneous distribution of the mutations) and further comparing the NGS-driven dPCR approach with traditional MRD methods, such as RT-qPCT and MFC.

In conclusion the NGS-driven dPCR approach is feasible for detecting, quantifying and monitoring gene mutations in AML and represents a promising advancement in MRD evaluation. Its ability to detect MRD even in the absence of markers tipically assessed by RT-qPCR and MFC, along with its higher sensitivity, makes it a valuable tool for refining MRD strategies and improving patient management.

8 – Figure index

(Clonal Hematopoiesis of Indeterminate Potential) may be present and contribute to the development of hematological malignancies.

Figure 11: *CHIP and risk of progressing to myeloid disease (shown on X-axis). AS mentioned above, certain factors that contribute to malignant transformation; (i) clonal size (and VAF), shown on the y-axis (ii) acquisition of additional somatic mutations, (iii) concomitant somatic mutations in specific genes (IDH1, IDH2, spliceosome genes mutations and TP53) and (iv) increase in morphological features of dysplasia. HPSC: hematopoietic stem and progenitor cell; CHIP: Clonal Hematopoiesis of Indeterminate Significance; CCUS: Clonal Cytopenia of Undetermined Significance; MDS: Myelodysplastic Syndrome; AML: Acute Myeloid Leukemia; From Gondek L.P. et al*[116]*.*

Figure 12: *Comparison between normal hematopoiesis (A) and malignant hematopoiesis (B), highlighted in the red box. Two possible scenarios are presented: in the first one, HSC acquired mutations (MUT1a; MUT2; MUT3) and subsequently becomes LSC with unlimited self-reneval (indicated by circle arrow); in the second one a MPP cells acquires a mutation (MUT1b) which restores the cell's capacity for self-renewal and then, as has been seen in HSC, acquires further mutations (MUT2; MUT3). The resulting Leukemia Stem Cell gives rise to blast cells. These have lost their capacity for self-renewal, and will not produce functional cells, despite the mimicry of normal hematopoiesis. HSC: Hematopoietic Stem Cell; MPP: Multipotent Progenitor; CMP: Common Myeloid Progenitor; CLP: Common Lymphoid Progenitor; MP: Megakaryocyte Progenitor; GMP: macrophage granulocytic progenitor; EP: Erythropoietin Progenitor; LSC: Leukemia Stem Cell. Adapted from Vagapova E.R. et al.*[77]*.*

Figure 13*: Study flowchart.*

Figure 14: dPCR workflow used to monitor MRD in AML patients over multiple time points. Peripheral blood, bone marrow and exosomes were analyzed to detect and quantify gene mutations with high sensitivity and accuracy.

Figure 15: Flowchart illustrates the enrolled and analyzed patients and their progression through the study. The chart highlights the number of patients at each timepoint.

Figure 16: Box A represents the frequency of markers detected at diagnosis. Among the patients analyzed, molecular markers were the most frequent (n = 7), followed by immunophenotypic and cytogenetic markers (n = 6 and 2, respectively). Each category represents the presence of at least one marker belonging to that class, regardless of any co-occurrence with other types. Box B represents the distribution of combinations of molecular,

immunophenotypic, and cytogenetic markers detected at diagnosis. Most patients had only one type of marker (n = 5).

Figure 17: Bar-plot illustrating the distribution of gene mutations identified in AML patients at diagnosis through NGS analysis. Each bar represents the number of patients with mutations in a specific gene.

Figure 18: Bar-plot illustrating all mutational events in genes identified in AML patients at diagnosis by NGS analysis.

Figure 19: Proportion of patients with at least one mutation identified via NGS and suitable for dPCR monitoring (A), and detectability of target mutations in both BM and PB (B); all selected mutations were simultaneously detected in BM and PB.

Figure 20: Custom versus commercial probes.

Figure 21: NGS-driven dPCR results in peripheral blood (A) and bone marrow (B) were highly concordant. Moreover, dPCR NGS-driven enabled MRD detection even in patients who lack conventional MRD markers.

Figure 22: dPCR NGS-driven detected residual leukemic cells when flow cytometry results were negative, providing earlier relapse prediction.

9 – Table index

10 – List of abbreviations

AML: Acute Myeloid Leukemia.

BM: Bone Marrow.

CNS: Central Nervous System.

BC: Blasts Cells.

MDS: Myelodysplastic Syndrome.

CBF: Core Binding Factor.

ICU: Intensive Care Unit.

CBC: Complete Blood Count.

EML: Extramedullary Leukemia.

CR: Complete Remission.

HR-MDS: high-risk MDS.

FC: Flow Cytometry.

IHC: Immunohistochemistry.

MPO: Myeloperoxidase.

FISH: Fluorescent in Situ Hybridization.

MRD: Measurable residual Disease.

RT-qPCR: Reverse Transcription Polymerase Chain Reaction.

NGS: Next Generation Sequencing.

WGS: Whole Genome Sequencing.

WES: Whole Exome Sequencing.

WTS: Whole Transcriptome Sequencing.

FAB: French-American-British.

WHO: World Health Organization.

ICC: International Consensus Classification.

ELN: Europen Leukemia Net.

CRi: Complete Remission with incomplete hematologic recovery.

MFLS: Morphologic Leukemia Free State.

KI: Kinase Inhibitor.

OS: Overall Survival.

EFS: Event Free Survival.

BsAbsB Bispecific Antibodies.

CAR-T: Chimeric Antigen Receptor T.

IWG: International Working Group.

PR: Partial Remission.

ANC: Absolute Neutrophil Count.

HSCs : Hematopoietic Stem Cells.

MPPx: Multipotent Progenitors.

CH: Clonal Hematopoiesis.

CHIP: Clonal Hematopoiesis of Indeterminate Significance.

ARCH: Age-Related Clonal Hematopoiesis.

CCUS: Clonal Cytopenia of Undetermined Significance.

ICUS: Idiopathic Cytopenias of Undetermined Significance.

CMUS: Clonal Monocytosis of Undetermined Significance.

VAF: Variant Allele Frequency.

LSC: Leukemic Stem Cell.

MFC: Multiparametric Flow Cytometry (MFC).

dPCR: Digital Polymerase Chain Reaction.

LOD: Limits of Detection.

LOQ: Limit of Quantification.

LAIP: Leukemia-associated immunophenotypes.

DfN: Different-from-Normal.

EOT: End of Treatment.

CSF: Cerebrospinal Fluid.

CTC: Circulating Tumor Cells.

cfDNA: Circulating Cell-Free DNA.

ctDNA: Circulating Tumor DNA.

cfRNA: Circulating Cell-Free RNA.

TEP: Tumor Instructed Platelets.

MVB: Multivesicular Body.

PMNs: Polymorphonuclear Leucocytes.

cdPCR: chip-based dPCR.

ddPCR: droplet-based dPCR.

IC: Informed Consent.

HSCT: Hematopoietic Stem Cell Transplantation.

DLI: Donor Lymphocyte Infusions.

HS: High Sensitivity.

11 – Bibliography

1. Devine SM, Larson RA. Acute leukemia in adults: recent developments in diagnosis and treatment. *CA Cancer J Clin*. 1994;44(6):326-52. doi:10.3322/canjclin.44.6.326
2. Jani CT, Ahmed A, Singh H, et al. Burden of AML, 1990-2019: Estimates From the Global Burden of Disease Study. *JCO Glob Oncol*. Sep 2023;9:e2300229. doi:10.1200/GO.23.00229
3. Liu H. Emerging agents and regimens for AML. *J Hematol Oncol*. Mar 23 2021;14(1):49. doi:10.1186/s13045-021-01062-w
4. Society AC. Acute Myeloid (myelogenous). American Cancer Society. http://www.cancer.org/cancer/leukemia-acutemyeloidaml/
5. Döhner H, Weisdorf DJ, Bloomfield CD. Acute Myeloid Leukemia. *N Engl J Med*. Sep 17 2015;373(12):1136-52. doi:10.1056/NEJMra1406184
6. Vakiti Anusha MP. Acute Myeloid Leukemia. In: LLC SP, ed. *MD Anderson manual of medical oncology*. 2023:1-25.
7. Heuser M, Ofran Y, Boissel N, et al. Acute myeloid leukaemia in adult patients: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol*. Jun 2020;31(6):697-712. doi:10.1016/j.annonc.2020.02.018
8. Abdallah M, Xie Z, Ready A, Manogna D, Mendler JH, Loh KP. Management of Acute Myeloid Leukemia (AML) in Older Patients. *Curr Oncol Rep*. Jul 28 2020;22(10):103. doi:10.1007/s11912-020-00964-1
9. Hasserjian RP, Steensma DP, Graubert TA, Ebert BL. Clonal hematopoiesis and measurable residual disease assessment in acute myeloid leukemia. *Blood*. May 14 2020;135(20):1729-1738. doi:10.1182/blood.2019004770
10. Stabellini N, Tomlinson B, Cullen J, et al. Sex differences in adults with acute myeloid leukemia and the impact of sex on overall survival. *Cancer Med*. Mar 2023;12(6):6711-6721. doi:10.1002/cam4.5461
11. De-Morgan A, Meggendorfer M, Haferlach C, Shlush L. Male predominance in AML is associated with specific preleukemic mutations. *Leukemia*. Mar 2021;35(3):867-870. doi:10.1038/s41375-020-0935-5
12. WHO WHO-. World Health Organization - WHO - Incidence, both sexes, in 2022. 2022.
13. Soulier J. Introduction to a review series on secondary leukemia. *Blood*. Jul 02 2020;136(1):1. doi:10.1182/blood.2019004171
14. Aquino VM. Acute myelogenous leukemia. *Curr Probl Pediatr Adolesc Health Care*. Feb 2002;32(2):50-8. doi:10.1067/mps.2002.121791
15. Kossman SE, Weiss MA. Acute myelogenous leukemia after exposure to strontium-89 for the treatment of adenocarcinoma of the prostate. *Cancer*. Feb 01 2000;88(3):620-4. doi:10.1002/(sici)1097-0142(20000201)88:3<620::aid-cncr19>3.0.co;2-#
16. Deschler B, Lübbert M. Acute myeloid leukemia: epidemiology and etiology. *Cancer*. Nov 01 2006;107(9):2099-107. doi:10.1002/cncr.22233
17. Poynter JN, Richardson M, Roesler M, et al. Chemical exposures and risk of acute myeloid leukemia and myelodysplastic syndromes in a population-based study. *Int J Cancer*. Jan 01 2017;140(1):23-33. doi:10.1002/ijc.30420
18. Tebbi CK. Etiology of Acute Leukemia: A Review. *Cancers (Basel)*. May 08 2021;13(9)doi:10.3390/cancers13092256
19. Li W. Leukemia. 2022.
20. Cheson BD, Bennett JM, Kopecky KJ, et al. Revised recommendations of the International Working Group for Diagnosis, Standardization of Response Criteria,

Treatment Outcomes, and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia. *J Clin Oncol*. Dec 15 2003;21(24):4642-9. doi:10.1200/JCO.2003.04.036
21. Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood*. May 19 2016;127(20):2391-405. doi:10.1182/blood-2016-03-643544
22. West AH, Godley LA, Churpek JE. Familial myelodysplastic syndrome/acute leukemia syndromes: a review and utility for translational investigations. *Ann N Y Acad Sci*. Mar 2014;1310(1):111-8. doi:10.1111/nyas.12346
23. Sandler DP, Collman GW. Cytogenetic and environmental factors in the etiology of the acute leukemias in adults. *Am J Epidemiol*. Dec 1987;126(6):1017-32. doi:10.1093/oxfordjournals.aje.a114741
24. Ley TJ, Mardis ER, Ding L, et al. DNA sequencing of a cytogenetically normal acute myeloid leukaemia genome. *Nature*. Nov 06 2008;456(7218):66-72. doi:10.1038/nature07485
25. Link DC. Molecular genetics of AML. *Best Pract Res Clin Haematol*. Dec 2012;25(4):409-14. doi:10.1016/j.beha.2012.10.002
26. Gilliland DG. Hematologic malignancies. *Curr Opin Hematol*. Jul 2001;8(4):189-91. doi:10.1097/00062752-200107000-00001
27. Reilly JT. Pathogenesis of acute myeloid leukaemia and inv(16)(p13;q22): a paradigm for understanding leukaemogenesis? *Br J Haematol*. Jan 2005;128(1):18-34. doi:10.1111/j.1365-2141.2004.05236.x
28. Kiyoi H NT, Nakano Y, Yokota S, Minami S, Miyawaki S, Asou N, Kuriyama K, Jinnai I, Shimazaki C, Akiyama H, Saito K, Oh H, Motoji T, Omoto E, Saito H, Ohno R, Ueda R. Prognostic implication of FLT3 and N-RAS gene mutations in acute myeloid leukemia. . *Blood*. 1999;
29. Valk PJ BD, Frew ME, Goodeve AC, Löwenberg B, Reilly JT. Second hit mutations in the RTK/RAS signaling pathway in acute myeloid leukemia with inv(16). . *Haematologica*. 2004;
30. Stirewalt DL, Kopecky KJ, Meshinchi S, et al. FLT3, RAS, and TP53 mutations in elderly patients with acute myeloid leukemia. *Blood*. Jun 01 2001;97(11):3589-95. doi:10.1182/blood.v97.11.3589
31. Higuchi M OBD, Kumaravelu P, Lenny N, Yeoh EJ, Downing JR. Expression of a conditional AML1-ETO oncogene bypasses embryonic lethality and establishes a murine model of human t(8;21) acute myeloid leukemia. *Cancer Cell*. 2022;
32. Newell LF, Cook RJ. Advances in acute myeloid leukemia. *BMJ*. Oct 06 2021;375:n2026. doi:10.1136/bmj.n2026
33. Stubbins RJ, Francis A, Kuchenbauer F, Sanford D. Management of Acute Myeloid Leukemia: A Review for General Practitioners in Oncology. *Curr Oncol*. Aug 30 2022;29(9):6245-6259. doi:10.3390/curroncol29090491
34. Siegal T, Benouaich-Amiel A, Bairey O. Neurologic complications of acute myeloid leukemia. Diagnostic approach and therapeutic modalities. *Blood Rev*. May 2022;53:100910. doi:10.1016/j.blre.2021.100910
35. Bakst RL, Tallman MS, Douer D, Yahalom J. How I treat extramedullary acute myeloid leukemia. *Blood*. Oct 06 2011;118(14):3785-93. doi:10.1182/blood-2011-04-347229
36. Ohanian M, Faderl S, Ravandi F, et al. Is acute myeloid leukemia a liquid tumor? *Int J Cancer*. Aug 01 2013;133(3):534-43. doi:10.1002/ijc.28012
37. Narayanan D, Weinberg OK. How I investigate acute myeloid leukemia. *Int J Lab Hematol*. Feb 2020;42(1):3-15. doi:10.1111/ijlh.13135

38. de Haas V, Ismaila N, Zhang L. Initial Diagnostic Workup of Acute Leukemia: ASCO Clinical Practice Guideline Endorsement Summary of the CAP and ASH Guideline. *J Oncol Pract*. Feb 2019;15(2):101-105. doi:10.1200/JOP.18.00613
39. Bennett JM, Catovsky D, Daniel MT, et al. Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. *Br J Haematol*. Aug 1976;33(4):451-8. doi:10.1111/j.1365-2141.1976.tb03563.x
40. Bene MC CG, Knapp W, Ludwig WD, Matutes E, Orfao A, van't Veer MB. Proposals for the immunological classification of acute leukemias. European Group for the Immunological Characterization of Leukemias (EGIL). *Leukemia*. 1995;
41. Khwaja A, Bjorkholm M, Gale RE, et al. Acute myeloid leukaemia. *Nat Rev Dis Primers*. Mar 10 2016;2:16010. doi:10.1038/nrdp.2016.10
42. Swerdlow SH CE, Harris NL , et al. *WHO*

Classification of Tumours of Haematopoietic and Lymphoid Tissues. 2017.
43. Azad A, Rajwa B, Pothen A. Immunophenotype Discovery, Hierarchical Organization, and Template-Based Classification of Flow Cytometry Samples. *Front Oncol*. 2016;6:188. doi:10.3389/fonc.2016.00188
44. Haferlach T, Schmidts I. The power and potential of integrated diagnostics in acute myeloid leukaemia. *Br J Haematol*. Jan 2020;188(1):36-48. doi:10.1111/bjh.16360
45. Jaffe E AD, Campo E , Harris NL , Quintanilla-Fend L . *Hematopathology*. 2nd ed . ed. 2016.
46. Dolnik A, Engelmann JC, Scharfenberger-Schmeer M, et al. Commonly altered genomic regions in acute myeloid leukemia are enriched for somatic mutations involved in chromatin remodeling and splicing. *Blood*. Nov 01 2012;120(18):e83-92. doi:10.1182/blood-2011-12-401471
47. Schiffer CA SRIKD, Pollock RE, Weichselbaum RR, et al. *Cancer medicine - Morphologic Classification and Clinical and Laboratory Correlate*. 6th edition ed. 2003.
48. G. A-H. *Classification of Acute Leukemia - Acute Leukemia - The Scientist's Perspective and Challenge*. Mariastefania Antica ed. 2011.
49. Bennett JM, Catovsky D, Daniel MT, et al. Proposal for the recognition of minimally differentiated acute myeloid leukaemia (AML-MO). *Br J Haematol*. Jul 1991;78(3):325-9. doi:10.1111/j.1365-2141.1991.tb04444.x
50. Vardiman JW, Harris NL, Brunning RD. The World Health Organization (WHO) classification of the myeloid neoplasms. *Blood*. Oct 01 2002;100(7):2292-302. doi:10.1182/blood-2002-04-1199
51. Alaggio R, Amador C, Anagnostopoulos I, et al. The 5th edition of the World Health Organization Classification of Haematolymphoid Tumours: Lymphoid Neoplasms. *Leukemia*. Jul 2022;36(7):1720-1748. doi:10.1038/s41375-022-01620-2
52. Marco Pizzi CGaAO. What's New in the Classification, Diagnosis and Therapy of Myeloid Leukemias. *Hemato*. 2023;
53. Arber DA, Orazi A, Hasserjian RP, et al. International Consensus Classification of Myeloid Neoplasms and Acute Leukemias: integrating morphologic, clinical, and genomic data. *Blood*. Sep 15 2022;140(11):1200-1228. doi:10.1182/blood.2022015850
54. Grob T, Al Hinai ASA, Sanders MA, et al. Molecular characterization of mutant TP53 acute myeloid leukemia and high-risk myelodysplastic syndrome. *Blood*. Apr 14 2022;139(15):2347-2354. doi:10.1182/blood.2021014472
55. Khoury JD, Solary E, Abla O, et al. The 5th edition of the World Health Organization Classification of Haematolymphoid Tumours: Myeloid and Histiocytic/Dendritic Neoplasms. *Leukemia*. Jul 2022;36(7):1703-1719. doi:10.1038/s41375-022-01613-1

56. Döhner H, Wei AH, Appelbaum FR, et al. Diagnosis and management of AML in adults: 2022 recommendations from an international expert panel on behalf of the ELN. *Blood*. Sep 22 2022;140(12):1345-1377. doi:10.1182/blood.2022016867
57. Papaemmanuil E, Gerstung M, Bullinger L, et al. Genomic Classification and Prognosis in Acute Myeloid Leukemia. *N Engl J Med*. Jun 09 2016;374(23):2209-2221. doi:10.1056/NEJMoa1516192
58. Kishtagari A, Levine RL, Viny AD. Driver mutations in acute myeloid leukemia. *Curr Opin Hematol*. Mar 2020;27(2):49-57. doi:10.1097/MOH.0000000000000567
59. Patel JP, Gönen M, Figueroa ME, et al. Prognostic relevance of integrated genetic profiling in acute myeloid leukemia. *N Engl J Med*. Mar 22 2012;366(12):1079-89. doi:10.1056/NEJMoa1112304
60. Ley TJ, Miller C, Ding L, et al. Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. *N Engl J Med*. May 30 2013;368(22):2059-74. doi:10.1056/NEJMoa1301689
61. Hou HA, Tien HF. Genomic landscape in acute myeloid leukemia and its implications in risk classification and targeted therapies. *J Biomed Sci*. Jul 21 2020;27(1):81. doi:10.1186/s12929-020-00674-7
62. De Kouchkovsky I, Abdul-Hay M. 'Acute myeloid leukemia: a comprehensive review and 2016 update'. *Blood Cancer J*. Jul 01 2016;6(7):e441. doi:10.1038/bcj.2016.50
63. Stone RM, Mandrekar SJ, Sanford BL, et al. Midostaurin plus Chemotherapy for Acute Myeloid Leukemia with a FLT3 Mutation. *N Engl J Med*. Aug 03 2017;377(5):454-464. doi:10.1056/NEJMoa1614359
64. Schaich M, Röllig C, Soucek S, et al. Cytarabine dose of 36 g/m² compared with 12 g/m² within first consolidation in acute myeloid leukemia: results of patients enrolled onto the prospective randomized AML96 study. *J Clin Oncol*. Jul 01 2011;29(19):2696-702. doi:10.1200/JCO.2010.33.7303
65. Löwenberg B, Pabst T, Vellenga E, et al. Cytarabine dose for acute myeloid leukemia. *N Engl J Med*. Mar 17 2011;364(11):1027-36. doi:10.1056/NEJMoa1010222
66. Burnett AK, Russell NH, Hills RK, et al. Optimization of chemotherapy for younger patients with acute myeloid leukemia: results of the medical research council AML15 trial. *J Clin Oncol*. Sep 20 2013;31(27):3360-8. doi:10.1200/JCO.2012.47.4874
67. Löwenberg B. Sense and nonsense of high-dose cytarabine for acute myeloid leukemia. *Blood*. Jan 03 2013;121(1):26-8. doi:10.1182/blood-2012-07-444851
68. Cornelissen JJ, Blaise D. Hematopoietic stem cell transplantation for patients with AML in first complete remission. *Blood*. Jan 07 2016;127(1):62-70. doi:10.1182/blood-2015-07-604546
69. Ferrara F, Picardi A. Is There Still a Role for Autologous Stem Cell Transplantation for the Treatment of Acute Myeloid Leukemia? *Cancers (Basel)*. Dec 24 2019;12(1)doi:10.3390/cancers12010059
70. Feldman EJ, Gergis U. Management of refractory acute myeloid leukemia: re-induction therapy or straight to transplantation? *Curr Hematol Malig Rep*. Mar 2012;7(1):74-7. doi:10.1007/s11899-011-0101-2
71. Döhner H, Estey E, Grimwade D, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood*. Jan 26 2017;129(4):424-447. doi:10.1182/blood-2016-08-733196
72. Heuser M, Freeman SD, Ossenkoppele GJ, et al. 2021 Update on MRD in acute myeloid leukemia: a consensus document from the European LeukemiaNet MRD Working Party. *Blood*. Dec 30 2021;138(26):2753-2767. doi:10.1182/blood.2021013626
73. HARRISON WJ. The total cellularity of the bone marrow in man. *J Clin Pathol*. May 1962;15(3):254-9. doi:10.1136/jcp.15.3.254

74. Hourigan CS, Gale RP, Gormley NJ, Ossenkoppele GJ, Walter RB. Measurable residual disease testing in acute myeloid leukaemia. *Leukemia*. Jul 2017;31(7):1482-1490. doi:10.1038/leu.2017.113
75. Teixeira A, Carreira L, Abalde-Cela S, et al. Current and Emerging Techniques for Diagnosis and MRD Detection in AML: A Comprehensive Narrative Review. *Cancers (Basel)*. Feb 21 2023;15(5)doi:10.3390/cancers15051362
76. Venditti A, Buccisano F, Del Poeta G, et al. Level of minimal residual disease after consolidation therapy predicts outcome in acute myeloid leukemia. *Blood*. Dec 01 2000;96(12):3948-52.
77. Ngai LL, Kelder A, Janssen JJWM, Ossenkoppele GJ, Cloos J. MRD Tailored Therapy in AML: What We Have Learned So Far. *Front Oncol*. 2020;10:603636. doi:10.3389/fonc.2020.603636
78. Schuurhuis GJ, Heuser M, Freeman S, et al. Minimal/measurable residual disease in AML: a consensus document from the European LeukemiaNet MRD Working Party. *Blood*. Mar 22 2018;131(12):1275-1291. doi:10.1182/blood-2017-09-801498
79. Buckley SA, Appelbaum FR, Walter RB. Prognostic and therapeutic implications of minimal residual disease at the time of transplantation in acute leukemia. *Bone Marrow Transplant*. May 2013;48(5):630-41. doi:10.1038/bmt.2012.139
80. Georgi JA, Stasik S, Bornhäuser M, Platzbecker U, Thiede C. Analysis of Subset Chimerism for MRD-Detection and Pre-Emptive Treatment in AML. *Front Oncol*. 2022;12:841608. doi:10.3389/fonc.2022.841608
81. Short NJ, Zhou S, Fu C, et al. Association of Measurable Residual Disease With Survival Outcomes in Patients With Acute Myeloid Leukemia: A Systematic Review and Meta-analysis. *JAMA Oncol*. Dec 01 2020;6(12):1890-1899. doi:10.1001/jamaoncol.2020.4600
82. Ossenkoppele G, Schuurhuis GJ. MRD in AML: does it already guide therapy decision-making? *Hematology Am Soc Hematol Educ Program*. Dec 02 2016;2016(1):356-365. doi:10.1182/asheducation-2016.1.356
83. Yu S, Huang F, Wang Y, et al. Haploidentical transplantation might have superior graft-versus-leukemia effect than HLA-matched sibling transplantation for high-risk acute myeloid leukemia in first complete remission: a prospective multicentre cohort study. *Leukemia*. May 2020;34(5):1433-1443. doi:10.1038/s41375-019-0686-3
84. Morsink LM, Sandmaier BM, Othus M, et al. Conditioning Intensity, Pre-Transplant Flow Cytometric Measurable Residual Disease, and Outcome in Adults with Acute Myeloid Leukemia Undergoing Allogeneic Hematopoietic Cell Transplantation. *Cancers (Basel)*. Aug 19 2020;12(9)doi:10.3390/cancers12092339
85. Hourigan CS, Dillon LW, Gui G, et al. Impact of Conditioning Intensity of Allogeneic Transplantation for Acute Myeloid Leukemia With Genomic Evidence of Residual Disease. *J Clin Oncol*. Apr 20 2020;38(12):1273-1283. doi:10.1200/JCO.19.03011
86. Moritz J, Schwab A, Reinisch A, Zebisch A, Sill H, Wölfler A. Measurable Residual Disease Detection in Acute Myeloid Leukemia: Current Challenges and Future Directions. *Biomedicines*. Mar 07 2024;12(3)doi:10.3390/biomedicines12030599
87. Vassault A, qualité mds-gMdl. [Interpretation and analysis of the requirements of the standard EN ISO 15189: 2012]. *Ann Biol Clin (Paris)*. Jun 2013;71 Spec No 1:19-27. doi:10.1684/abc.2013.0838
88. Buccisano F, Maurillo L, Schuurhuis GJ, et al. The emerging role of measurable residual disease detection in AML in morphologic remission. *Semin Hematol*. Apr 2019;56(2):125-130. doi:10.1053/j.seminhematol.2018.09.001

89. Chea M, Rigolot L, Canali A, Vergez F. Minimal Residual Disease in Acute Myeloid Leukemia: Old and New Concepts. *Int J Mol Sci*. Feb 10 2024;25(4)doi:10.3390/ijms25042150
90. Chatterjee T, Mallhi RS, Venkatesan S. Minimal residual disease detection using flow cytometry: Applications in acute leukemia. *Med J Armed Forces India*. Apr 2016;72(2):152-6. doi:10.1016/j.mjafi.2016.02.002
91. Ding L, Ley TJ, Larson DE, et al. Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. *Nature*. Jan 11 2012;481(7382):506-10. doi:10.1038/nature10738
92. Voso MT, Ottone T, Lavorgna S, et al. MRD in AML: The Role of New Techniques. *Front Oncol*. 2019;9:655. doi:10.3389/fonc.2019.00655
93. Yoest JM, Shirai CL, Duncavage EJ. Sequencing-Based Measurable Residual Disease Testing in Acute Myeloid Leukemia. *Front Cell Dev Biol*. 2020;8:249. doi:10.3389/fcell.2020.00249
94. Welch JS, Ley TJ, Link DC, et al. The origin and evolution of mutations in acute myeloid leukemia. *Cell*. Jul 20 2012;150(2):264-78. doi:10.1016/j.cell.2012.06.023
95. Jongen-Lavrencic M, Grob T, Hanekamp D, et al. Molecular Minimal Residual Disease in Acute Myeloid Leukemia. *N Engl J Med*. Mar 29 2018;378(13):1189-1199. doi:10.1056/NEJMoa1716863
96. Heuser M, Heida B, Büttner K, et al. Posttransplantation MRD monitoring in patients with AML by next-generation sequencing using DTA and non-DTA mutations. *Blood Adv*. May 11 2021;5(9):2294-2304. doi:10.1182/bloodadvances.2021004367
97. Meddi E, Savi A, Moretti F, et al. Measurable Residual Disease (MRD) as a Surrogate Efficacy-Response Biomarker in AML. *Int J Mol Sci*. Feb 04 2023;24(4)doi:10.3390/ijms24043062
98. Parkin B, Londoño-Joshi A, Kang Q, Tewari M, Rhim AD, Malek SN. Ultrasensitive mutation detection identifies rare residual cells causing acute myelogenous leukemia relapse. *J Clin Invest*. Sep 01 2017;127(9):3484-3495. doi:10.1172/JCI91964
99. Terwijn M, van Putten WL, Kelder A, et al. High prognostic impact of flow cytometric minimal residual disease detection in acute myeloid leukemia: data from the HOVON/SAKK AML 42A study. *J Clin Oncol*. Nov 01 2013;31(31):3889-97. doi:10.1200/JCO.2012.45.9628
100. Spangrude GJ, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. *Science*. Jul 01 1988;241(4861):58-62. doi:10.1126/science.2898810
101. Akashi K, Traver D, Miyamoto T, Weissman IL. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature*. Mar 09 2000;404(6774):193-7. doi:10.1038/35004599
102. Kondo M, Weissman IL, Akashi K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell*. Nov 28 1997;91(5):661-72. doi:10.1016/s0092-8674(00)80453-5
103. Hu M, Krause D, Greaves M, et al. Multilineage gene expression precedes commitment in the hemopoietic system. *Genes Dev*. Mar 15 1997;11(6):774-85. doi:10.1101/gad.11.6.774
104. Vagapova ER, Spirin PV, Lebedev TD, Prassolov VS. The Role of TAL1 in Hematopoiesis and Leukemogenesis. *Acta Naturae*. 2018;10(1):15-23.
105. Libby P, Sidlow R, Lin AE, et al. Clonal Hematopoiesis: Crossroads of Aging, Cardiovascular Disease, and Cancer: JACC Review Topic of the Week. *J Am Coll Cardiol*. Jul 30 2019;74(4):567-577. doi:10.1016/j.jacc.2019.06.007

106. Martincorena I, Campbell PJ. Somatic mutation in cancer and normal cells. *Science*. Sep 25 2015;349(6255):1483-9. doi:10.1126/science.aab4082
107. Jaiswal S, Fontanillas P, Flannick J, et al. Age-related clonal hematopoiesis associated with adverse outcomes. *N Engl J Med*. Dec 25 2014;371(26):2488-98. doi:10.1056/NEJMoa1408617
108. Genovese G, Kähler AK, Handsaker RE, et al. Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. *N Engl J Med*. Dec 25 2014;371(26):2477-87. doi:10.1056/NEJMoa1409405
109. Malcovati L, Cazzola M. The shadowlands of MDS: idiopathic cytopenias of undetermined significance (ICUS) and clonal hematopoiesis of indeterminate potential (CHIP). *Hematology Am Soc Hematol Educ Program*. 2015;2015:299-307. doi:10.1182/asheducation-2015.1.299
110. Köhnke T, Majeti R. Clonal Hematopoiesis: From Mechanisms to Clinical Intervention. *Cancer Discov*. Dec 01 2021;11(12):2987-2997. doi:10.1158/2159-8290.CD-21-0901
111. Bejar R. Myelodysplastic Syndromes Diagnosis: What Is the Role of Molecular Testing? *Curr Hematol Malig Rep*. Sep 2015;10(3):282-91. doi:10.1007/s11899-015-0270-5
112. Valent P, Orazi A, Steensma DP, et al. Proposed minimal diagnostic criteria for myelodysplastic syndromes (MDS) and potential pre-MDS conditions. *Oncotarget*. Sep 26 2017;8(43):73483-73500. doi:10.18632/oncotarget.19008
113. Younes IE, Syler L, Hamed A. Review of clonal hematopoiesis, subtypes and its role in neoplasia and different morbidities. *Leuk Res*. Jul 2023;130:107307. doi:10.1016/j.leukres.2023.107307
114. Asada S, Kitamura T. Clonal hematopoiesis and associated diseases: A review of recent findings. *Cancer Sci*. Oct 2021;112(10):3962-3971. doi:10.1111/cas.15094
115. Steensma DP, Bejar R, Jaiswal S, et al. Clonal hematopoiesis of indeterminate potential and its distinction from myelodysplastic syndromes. *Blood*. Jul 02 2015;126(1):9-16. doi:10.1182/blood-2015-03-631747
116. Gondek LP, DeZern AE. Assessing clonal haematopoiesis: clinical burdens and benefits of diagnosing myelodysplastic syndrome precursor states. *Lancet Haematol*. Jan 2020;7(1):e73-e81. doi:10.1016/S2352-3026(19)30211-X
117. Heuser M, Thol F, Ganser A. Clonal Hematopoiesis of Indeterminate Potential. *Dtsch Arztebl Int*. May 06 2016;113(18):317-22. doi:10.3238/arztebl.2016.0317
118. Xie M, Lu C, Wang J, et al. Age-related mutations associated with clonal hematopoietic expansion and malignancies. *Nat Med*. Dec 2014;20(12):1472-8. doi:10.1038/nm.3733
119. Desai P, Hassane D, Roboz GJ. Clonal Hematopoiesis and risk of Acute Myeloid Leukemia. *Best Pract Res Clin Haematol*. Jun 2019;32(2):177-185. doi:10.1016/j.beha.2019.05.007
120. Abelson S, Collord G, Ng SWK, et al. Prediction of acute myeloid leukaemia risk in healthy individuals. *Nature*. Jul 2018;559(7714):400-404. doi:10.1038/s41586-018-0317-6
121. Desai P, Mencia-Trinchant N, Savenkov O, et al. Somatic mutations precede acute myeloid leukemia years before diagnosis. *Nat Med*. Jul 2018;24(7):1015-1023. doi:10.1038/s41591-018-0081-z
122. Buccisano F, Maurillo L, Gattei V, et al. The kinetics of reduction of minimal residual disease impacts on duration of response and survival of patients with acute myeloid leukemia. *Leukemia*. Oct 2006;20(10):1783-9. doi:10.1038/sj.leu.2404313
123. Nakao M, Yokota S, Iwai T, et al. Internal tandem duplication of the flt3 gene found in acute myeloid leukemia. *Leukemia*. Dec 1996;10(12):1911-8.

124. Yamamoto Y, Kiyoi H, Nakano Y, et al. Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. *Blood*. Apr 15 2001;97(8):2434-9. doi:10.1182/blood.v97.8.2434
125. Cilloni D, Renneville A, Hermitte F, et al. Real-time quantitative polymerase chain reaction detection of minimal residual disease by standardized WT1 assay to enhance risk stratification in acute myeloid leukemia: a European LeukemiaNet study. *J Clin Oncol*. Nov 01 2009;27(31):5195-201. doi:10.1200/JCO.2009.22.4865
126. Böyum A. Isolation of mononuclear cells and granulocytes from human blood. Isolation of monuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. *Scand J Clin Lab Invest Suppl*. 1968;97:77-89.
127. Quan PL SM, Brouzes E. dPCR: A Technology Review. *Sensors* 2018;
128. Coccaro N, Tota G, Anelli L, Zagaria A, Specchia G, Albano F. Digital PCR: A Reliable Tool for Analyzing and Monitoring Hematologic Malignancies. *Int J Mol Sci*. Apr 29 2020;21(9)doi:10.3390/ijms21093141