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Biomarkers in acute myeloid leukemia: From state of the art in risk classification to future challenges of RNA editing as disease predictor and therapy target

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ABSTRACT

Acute Myeloid Leukemia (AML) is currently diagnosed based on morphological assessment of myeloid cells' features, immunophenotypic characterization of specific cell surface and intracellular markers, conventional cytogenetic testing and screening for genetic abnormalities in bone marrow and peripheral blood specimens. In recent years new technologies have shed light on the complexity and heterogeneity of this elusive leukemia and are providing useful biomarkers, predictive of prognosis in AML patients. Hence, technological efforts are being made in order to identify more accurate AML biomarkers also useful to track minimal residual disease at the various follow-up times. This remains an unmet need that, together with the intrinsic tumor features of AML, results in the highest death rate of all leukemias and a 5-year overall survival <50%. This review provides insights into the state-of-the-art of AML-related biomarkers and their role in clinical practice as prognostic indicators, minimal residual disease detection or candidates for targeted therapy. In addition, we report modifications of RNA epitranscriptome during normal hematopoiesis that are de-regulated in AML, recently revealed by new and more sophisticated techniques. We focus on alterations of m^{6}A modifications on mRNAs and of enzymes catalyzing them, which have been reported to affect normal hematopoiesis and leukemogenesis and are providing novel promising biomarkers for AML risk assessment and newly druggable targets for treatment.

1. Introduction

Acute Myeloid Leukemia (AML) is a bone marrow (BM) disease that arises from the abnormal expansion and maturation of genetically aberrant hematopoietic stem/progenitor cells (HSPCs) (Kantarjian et al., 2021). These cells display a proliferative advantage over normal HSPCs, leading to impaired normal hematopoiesis and triggering BM failure and cytopenia. AML pathophysiology, although not completely clarified, is related to various genetic and epigenetic changes in HSPCs, causing abnormalities in hematopoietic cell proliferation, maturation/differentiation and survival. In recent years new technologies have shed new light on the complexity and heterogeneity of this elusive leukemia and have provided valuable biomarkers, predictive of prognosis in AML patients.

Despite several new treatments have been approved recently for clinical trials, AML remains a highly lethal disease, having the highest death rate among leukemias with a 5-year overall survival (OS) < 50% (Kantarjian et al., 2021). The updated 2022 European Leukemia Net (ELN) risk classification (Dohner et al., 2022) considers the most recurrent genetic abnormalities to predict prognosis. However, several AMLs cases classified as intermediate risk lack specific biomarkers, resulting in less defined clinical indications for prognosis and therapy.

An additional confounding factor is that certain genetic aberrations

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Abbreviations

3'UTR	3'-untranslated regions
ALKBH5	AlkB Homolog 5
AML	Acute Myeloid Leukemia
APL	Acute Promyelocytic Leukemia
ASXL1	Additional Sex Combs like 1
BM	Bone Marrow
bZIP	basic leucine zipper
CBF	Core-Binding Factor
CBFB	core-binding factor subunit beta
CEBPA	CCAAT/enhancer-binding protein-alpha
CHIP	Clonal Hematopoiesis of Indeterminate Potential
CR	Complete Responses
ddPCR	droplet digital PCR
DNMT3A	DNA methyltransferase 3 alpha
FLT3	FMS-like tyrosine kinase 3
FTO	fat-mass- and obesity-associated protein
ELN	European Leukemia Net
FISH	Fluorescence In Situ Hybridization
HSPC	Hematopoietic Stem/Progenitor Cells
IDH1/2	Isocitrate Dehydrogenase 1/2
ITD	Internal Tandem Duplication
KMT2A	lysine methyltransferase 2A
LLPS	Liquid-Liquid Phase Separation
m^6A	N6-methyladenosine
MACOM	METTL-Associated Complex
METTL3	methyltransferase-like 3
METTL14	methyltransferase-like 14
miRNAs	MicroRNAs
MRD	Minimal Residual Disease
MTD	methyltransferase domain
lncRNA	long non-coding RNA
NPM1	Nucleophosmin
OS	Overall Survival
PML	PML nuclear body scaffold
RARA	Retinoic Acid Receptor Alpha
RUNX1	RUNX family transcription factor 1
RQ-PCR	Real-time Quantitative Polymerase Chain Reaction
SAM	S-adenosy-L-methionine
TET2	Ten-Eleven-Translocation 2
TKD	Tyrosine Kinase Domain
MFC	Multiparameter Flow Cytometry
MDS	Myelodysplastic Syndromes
WHO	World Health Organization
WT1	Wilms' tumor 1

affecting HSPCs (*e.g.*, *DNMT3A*, *TET2*, *ASXL1*, *IDH1* and *IDH2* mutations) emerge as a function of age and are not directly connected to leukemia. These hits define a pre-leukemic state (namely Clonal Hematopoiesis of Indeterminate Potential – CHIP), conferring a proliferative advantage, in the absence of an overt disease (Steensma, 2018; Zviran et al., 2020). Clonal expansion of CHIP-related subclones may occur when they acquire further AML-defining mutations. Hence, pre-leukemic aberrations may not be ideal targets for tracking disease evolution and for therapy.

Furthermore, the increasing power of techniques like RNA-seq is revealing that specific gene expression profiles could be associated with the mutational status of AML, helping in defining risk categories (Wang et al., 2017). Nevertheless, the 5-years overall survival and relapse frequency in AML are still discouraging compared to other leukemias, suggesting that more efforts should be lavished to ameliorate AML patients' outcome (Kantarjian et al., 2021). After induction chemotherapy, the persistence of tumor cells in the BM below the morphologic detection threshold is often the cause of clonal selection, proliferation and eventually relapse. Consequently, measuring leukemia-specific genetic or immunophenotypic traces (namely Minimal Residual Disease - MRD), has become crucial in the clinical management of AML patients (Gorello et al., 2006; Lambert et al., 2014; Schnittger et al., 2009), and has shown to provide valuable prognostic information.

2. Features of optimal new AML biomarkers

Individuating novel prognostic markers in AML is not trivial, as several variables must be included in the studied model of patient cohorts, to demonstrate the independence of risk predictors. Useful cues might emerge from genes that are highly expressed in other tumors. The development of large platforms annotating data by Next Generation Sequencing (NGS) in cancer patients, such as The Cancer Genome Atlas (TGCA) (Cancer Genome Atlas Research et al., 2013) or Gene Expression Omnibus (GEO) (Edgar et al., 2002), allows the immediate availability of expression levels data, often with clinically relevant information. For example, among fucosyl-transferase family (FUT) genes, the high expression of genes *FUT3*, *FUT6* and *FUT7* is associated to adverse effect on event-free and overall survival in AML patients receiving chemotherapy. However, only *FUT3* preserves its predictive potential in patients undergoing allogeneic Hematopoietic Stem Cell Transplantation (Allo-HSCT) (Dai et al., 2020). This further confirms the importance of timing in the advocated employment of such markers.

Notably, TGCA or other available platforms for gene expression data allows to replicate the findings observed in a novel studied AML patient cohort, thus, when possible, reciprocally confirming the reliability of the results. The importance of confirming data extracted from repository in extensively studied AML cohorts is supported by the fact that often database records lack of important clinical information, such as progression/relapse or treatment/therapy, useful to appropriately stratify patients (Mehta et al., 2022).

Despite the preciseness of bioinformatics-based approaches, there are considerable caveats in adopting the gene expression levels as biomarkers in AML risk stratification. Two hurdles in this sense are represented by the standardization of measurement methods and the definition of what is considered "high" or "low" expression, according to rigorous models of statistical analysis (Raman et al., 2019). Stratifying expression levels of the studied cohort or dataset in ranges could be a more accurate strategy (Raman et al., 2019). However, it is common to divide them in only two groups according to the mean or median, and subsequently to perform regression-models using covariates (Smith and Sheltzer, 2022).

Noteworthy, the variance of the expression values within the observed cohort might be important in choosing the candidate biomarker, as a higher variance could correspond to a higher discrimination capability between different prognoses (Rázga and Némethová, 2017). Ultimately, the research for new prognostic biomarkers should also include genes not relevant as therapeutic targets, since it has recently been proved that the expression of main cancer driver genes is not systematically associated with outcomes (Smith and Sheltzer, 2022).

3. Techniques to identify novel biomarkers and to detect minimal residual disease in AML

Detection of AML biomarkers can be achieved through several techniques, including cytogenetic studies (*e.g.*, karyotyping, Fluorescence In Situ Hybridization-FISH), immunophenotyping (*e.g.*, via Multiparameter Flow Cytometry-MFC) and molecular genetic methods (*e.g.*, real-time quantitative polymerase chain reaction-RQ-PCR, droplet

digital PCR-ddPCR, and NGS). These procedures characterize the features of each individual AML at diagnosis and trace them over their clinical course to assess response to therapy and prognosis (Greenberg et al., 2012; He et al., 2015).

3.1. Cytogenetic analysis

Myeloid cells readily divide in *vitro* cultures; hence chromosome banding has been successfully used for years in the context of myeloid malignancies (Michaeli et al., 1986). Chromosome banding analysis has been implemented in the clinical work-up of hematological malignancies to identify genome-wide numerical and structural aberrations, and to perform risk stratifications. Moreover, the development of FISH techniques introduced the advantage of not requiring dividing cells and detecting abnormalities smaller than 10 Mb, thus improving the resolution of the cytogenetic studies (He et al., 2015). FISH is now widely used in myeloid malignancies since it can detect most of the AML-specific abnormalities (He et al., 2015). For these reasons, cytogenetic analysis is pivotal in the diagnosis of AML (He et al., 2015), and it is included in the most recent clinical guidelines of ELN, National Comprehensive Cancer Network (NCCN) and the Revised International Prognostic Scoring System (IPSS-R) (Greenberg et al., 2012).

3.2. Immunophenotype

This method relies on the detection of leukemia-associated immunophenotypes (LAIP), which can be identified at diagnosis through a combination of normal myeloid progenitors and aberrant surface markers in BM aspirated or Peripheral Blood (PB) cells (Terwijn et al., 2013). LAIPs are then monitored at follow-up and compared to diagnosis (Zeijlemaker et al., 2019) or they can be profiled directly on follow-up samples using the difference-from-normal approach (Wood, 2020). MFC is widely available in many centers and currently represents the gold-standard to determine MRD in AML, with sensitivities ranging from 10–3 to 10–5 (Feller et al., 2004). However, hurdles of MFC are the standardization of flow-cytometry parameters, data analysis and interpretation, which make challenging to compare data from different centers (Tettero et al., 2022).

3.3. RQ-PCR and ddPCR

Molecular genetic methods mainly rely on RQ-PCR to detect fusion transcripts or specific somatic mutations in AML with high sensitivity thresholds (down to 10-5-10-6) (Hokland and Ommen, 2011). Example of biomarkers that can be reliably detected by RQ-PCR are: *PML*::*RARA*; *RUNX1*::*RUNX1T1*; *CBFB*::*MYH11* gene fusions, *NPM1* mutations (Gorello et al., 2006), *WT1* mutations (Cilloni et al., 2009), particularly when combined with MFC (Malagola et al., 2016). The use of ddPCR has improved quantification by increasing sensitivity in detecting low target copies, thus reducing the amount of patient's diagnostic samples, and overcoming the normalization issue. This approach has proven successful in the detection of *NPM1* mutations (Bacher et al., 2014; Bill et al., 2018) and other leukemia-associated mutations, such as isocitrate dehydrogenases 1/2 (*IDH1/2*) mutations (Grassi et al., 2020). However, a major hurdle for molecular MRD detection by ddPCR, as RQ-PCR, is that assays for each mutation must be specifically designed, thus leaving other biomarkers undetectable.

3.4. NGS

The emergence of new techniques is allowing more and more sensitive assessment of MRD states. NGS is a high-throughput sequencing technique with different capabilities ranging from targeted approaches to Whole-Exome Sequencing (WES) or Whole-Genome Sequencing (WGS). NGS allows the simultaneous detection of different patient-specific gene mutations in a single assay (Yoest et al., 2020).

Many studies have implemented NGS as a tool to identify AML biomarkers and trace them over time with powerful prognostic implications (Vonk et al., 2021). Early evidences demonstrated that NGS could reliably trace specific genetic aberrations, such as *NPM1*, *FLT3*-ITD mutations (Thol et al., 2012), and *RUNX1* mutations (Kohlmann et al., 2014). Moreover, this technique allows the screening of larger panels of AML-related genes (Cappelli et al., 2022). Although limited, the studies combining current NGS methods and MFC to detect MRD in AMLs showed an overall concordance rate of about 70–80%. Patients found MRD + by only one of the two approaches have a worse outcome compared to patients MRD-for both assays (Getta et al., 2017; Guolo et al., 2017; Jongen-Lavrencic et al., 2018; Patkar et al., 2021). A recent study conducted on 201 AML patients evaluated for MRD after induction and consolidation chemotherapy reported a superior role for NGS in predicting clinical outcome compared to MFC (Patkar et al., 2021). However, NGS is still presenting important limiting factors. These include error rates, partially solved with the introduction of Unique-Molecular Barcodes-UMIs (Pfeiffer et al., 2018; Salk et al., 2018). Other limitations are sensitivity and specificity rates, depending on the region of interest and on the width of the assay used, (with WES and WGS having much lower sensitivities than targeted sequencing), as well as costly reagents/equipment and specialized personnel.

However, MRD genetic markers are not always stable over the course of AML and some of them can be related to a preleukemic state (Corces-Zimmerman et al., 2014; Shlush et al., 2014). Thus, it is still necessary to pinpoint molecular targets that are leukemic cells' specific and related to a proliferative advantage ultimately leading to relapse (Steensma, 2018).

3.5. Epigenetic landscape

Another promising approach exploiting NGS is the characterization of the epigenetic landscape in AMLs for the identification of broad DNA and chromatin modifications specific to the disease. The genome-wide analysis of DNA methylation and functional studies have shown that aberrant methylation patterns are often detected across the AML genome and contribute to tumorigenesis (Božić et al., 2022). Interestingly, several AML-related mutations have been detected in genes whose products are involved in the epigenetic modification of DNA or chromatin structure and transcriptional gene regulation, such as *DNMT3A*, *IDH1*, *IDH2* and *TET2* (Božić et al., 2022; Figueroa et al., 2010). However, the clinical impact of these findings on AML prognosis is still controversial (Božić et al., 2022).

3.6. Innovative technologies

The above-mentioned assays are performed on the AML bulk, which included a mixed population composed of AML blasts and normal peripheral blood or BM cells. Bulk sequencing relies on averaged gene expression from a population of cells, not allowing the reconstruction of the complex architecture of clonal evolution of AML or the identification of specific culprits for clonal selection and relapse. Therefore, novel single-cell sequencing techniques are emerging as powerful tools to define the molecular landscape of cell clones and their changes over the patients' clinical history. Although this technology is developing rapidly, it is still at its infancy and is far from being implemented in clinical routine, due to the high costs and difficulties in data processing and analysis.

4. Biomarkers currently adopted in clinical practice for AML risk classification

The World Health Organization (WHO) 2022 classification of myeloid neoplasms defines different AML entities based on a combination of various features, including morphology, immunophenotyping, cytogenetics and molecular aberrations (Khoury et al., 2022) (Table 1).

Table 1
WHO 2022 classification of AML.

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

In the newest WHO classification, the threshold of the blasts percentage for AML with defining genetic abnormalities, (including *PML*::*RARA*; *RUNX1*::*RUNX1T1*; *CBFB*::*MYH11*; *DEK*::*NUP214*), has been reduced from >20% to >10%. The AML subtypes defined by differentiation include the cases without defining genetic biomarkers. A more comprehensive genetic characterization of these AMLs is expected to decrease their number in the near future.

In 2022, the ELN released an update of the AML classification and a revised ELN genetic risk classification (Dohner et al., 2022), providing physicians with a list of biomarkers to be assessed when approaching a newly diagnosed AML patient (Table 2). Chromosomal aberrations have shown to be useful prognostic markers in AMLs (Mrózek et al., 2001), therefore they are used to categorize AML in three risk groups: favorable, intermediate and adverse.

The *favorable ELN risk group* includes AMLs carrying specific genetic abnormalities: t(8;21) (q22;q22)/*RUNX1*::*RUNX1T1*, inv(16) (p13q22) and t(16;16)(p13;q22)/*CBF*::*MYH11*, which involve genes encoding the components of the Core-Binding Factor (CBF) complex, hence defining a historical AML subtype named CBF-AML. CBF-AMLs are associated with the most favorable response to conventional anthracycline- and cytarabine-based induction chemotherapy (up to 90% complete responses - CR), prolonged disease remission and survival (Döhner et al., 2017). Therapies with fludarabine, cytarabine, granulocyte colony-stimulating factor (FLAG), and idarubicin (FLAG-Ida), further improve the responses of CBF-AMLs, with survival rates of 95% after 8 years (Burnett et al., 2013). The addition of gemtuzumab ozogamicin to FLAG regimen has showed similar promising results (Borthakur et al., 2014).

Table 2
European Leukemia Network genetic risk classification- 2022 update.

Risk category	Genetic abnormality
Favorable	t(8;21)(q22;q22.1)/*RUNX1::RUNX1T1*
	inv(16)(p13.1q22) or t(16;16)(p13.1;q22)/*CBFB::MYH11*
	Mutated *NPM1* without *FLT3*-ITD
	bZIP in-frame mutated *CEBPA*
Intermediate	Mutated *NPM1* with *FLT3*-ITD
	Wild-type *NPM1* with *FLT3*-ITD
	t(9;11)(p21.3;q23.3)/*MLLT3::KMT2A*
	Cytogenetic and/or molecular abnormalities not classified as favorable or adverse
Adverse	t(6;9)(p23;q34.1)/*DEK::NUP214*
	t(v;11q23.3)/*KMT2A*-rearranged
	t(9;22)(q34.1;q11.2)/*BCR::ABL1*
	t(8;16)(p11;p13)/*KAT6A::CREBBP*
	inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2)/*GATA2, MECOM(EVI1)*
	t(3q26.2;v)/*MECOM(EVI1)*-rearranged
	−5 or del(5q); −7; −17/abn(17p)
	Complex karyotype, monosomal karyotype
	Mutated *ASXL1*, *BCOR, EZH2, RUNX1, SF3B1, SRSF2, STAG2, U2AF1* or *ZRSR2*
	Mutated *TP53*

The *intermediate ELN risk group includes* AMLs bearing *FLT3*-ITD (with or without *NPM1* mutation) and t(9;11)(p21.3;q23.3)/*MLLT3*::*KMT2A*. AMLs with normal karyotype, cytogenetic and/or molecular abnormalities not classified as favorable or adverse, are also included in this group.

In *the adverse ELN risk group* are included AML bearing cytogenetic abnormalities as: t(6;9)(p23;q34.1)/*DEK*::*NUP214*; t(v;11q23.3)/*KMT2A*-rearranged, t(9;22)(q34.1;q11.2)/*BCR*::*ABL1*; t(8;16)(p11;p13)/*KAT6A*::*CREBBP*, t(3q26.2;v)/*MECOM*(*EVI1*)-rearranged; inv (3)(q21q26) or t(3;3)(q21;q26)/*GATA2*; *MECOM*(*EVI1*), −5 or del(5q); −7; abn(17p) or complex karyotype (defined as ≥3 chromosomal abnormalities in the absence of one of the WHO designated recurring translocations or inversions), which show poor outcome with increased risk of relapse (Grimwade et al., 2010).

4.1. AML with t(6;9)(p23;q34.1)/DEK-NUP214

The resulting chimeric fusion protein between nuclear phosphoprotein DEK and nucleoporin NUP214 (previously named as CAN), acts as a pro-tumorigenic factor (von Lindern et al., 1992), and increases protein anabolic processes in myeloid cells (Ageberg et al., 2008). AML, t(6;9) is present in a small subgroup of patients (1–2%), and it is usually associated with mutations in *FLT3* gene (~90%) (Slovak et al., 2006) and with poor 5-year OS (9%–28%) (Slovak et al., 2006).

4.2. AML with inv(3)(q21.3q26.2) or t(3;3) (q21.3;q26.2)/GATA2, MECOM

This fusion juxtaposes the *GATA2* enhancer to the *MECOM* (*EVI1*) gene, activating its expression and producing *GATA2* haploinsufficiency (Gröschel et al., 2014). This anomaly is rarely detected in AML (1–2%) and it is mostly associated with other chromosomal abnormalities and gene mutations of the RAS pathway (Gröschel et al., 2014)(see below). No specific treatment for this entity has been identified and less than 30% of this patients' subgroup achieves a CR after intensive chemotherapy (Lugthart et al., 2010).

4.3. AML with BCR/ABL1

According to the most recent WHO classification, this is the only AML entity where the blast count (>20%) is still instrumental to distinguish AML from Chronic Myelogenous Leukemia (CML) in its initial blast phase. Therapy with Tyrosine Kinase Inhibitors (TKIs) is reasonable (Pompetti et al., 2007). However, due to the rarity of this entity, there is a lack of systematic clinical data.

4.4. AML with KMT2A-rearrangement

KMT2A rearrangements, formerly known as mixed lineage leukemia (*MLL1*) gene abnormalities, have been identified in ~10% of leukemias (Marschalek, 2016). This gene encodes for a histone methyltransferase regulating the expression of genes involved in hematopoiesis. AML bearing *KMT2A* rearrangements often show monocytic features and high blast counts. More than 80 *KMT2A* fusion partners have been described, with *MLLT3*, *AFDN*, *ELL*, and *MLLT10* being the most common. The identification of the fusion partner can provide prognostic information and potential therapeutic targets, although currently no specific treatments are available.

4.5. Acute promyelocytic leukemia (APL)

APL is characterized by the t(15;17)(q22;q12) translocation leading to the formation of the *PML-RARA* fusion gene, which represents an excellent APL biomarker deeply linked to the disease etiology that can be followed over time using RQ-PCR assays. The introduction of chemo-free regimes with all-trans retinoic acid and arsenic trioxide has proven to be highly effective in inducing APL blast differentiation/apoptosis, dramatically ameliorating the clinical course of APL patients (Sanz et al., 2019).

4.6. Gene mutations associated to AML

About 40–50% of AML patients do not show karyotype abnormalities. However, NGS studies are providing a spectrum of recurrent driver mutations that is instrumental to define the roots of leukemogenesis and can assist AML risk stratification. Mutations in *NPM1*, *FLT3*, *CEBPA*, *RUNX1*, *ASXL1* and *TP53* have a prognostic impact in AML and have been included in the ELN risk classification algorithm, reported above. The molecular characterization and genomic profiling of AML blasts is allowing the identification of patient-associated gene mutations, thus providing suitable candidates for the development of new targeted-therapies with the ultimate aim of personalized medicine. Table 3 summarizes the most relevant AML molecular targets and newer agents currently tested for the treatment of AML (Padmakumar et al., 2021).

4.7. NPM1 mutations

Nucleophosmin 1 (*NPM1*), is a nucleolar protein enriched in proliferating cells (Box et al., 2016). *NPM1* regulates vital cellular activities such as ribosome biogenesis, DNA repair and apoptosis. Mutations on this gene cause the protein to be aberrantly located in the cytoplasm preventing its normal shuttling to the nucleus (Verhaak et al., 2005), ultimately resulting in blockage of the ARF-p53 tumor suppressor pathway controlling cell proliferation and apoptosis (Falini et al., 2005). At present, more than 50 different *NPM1* mutations have been identified, with subtypes A, B and D being the most frequent (Falini et al., 2009). *NPM1* mutations have been described as a late, disease-defining leukemogenic event, giving rise to a specific AML subtype (around 30% of all AMLs) (Thiede et al., 2006). This entity is associated with normal karyotype (~50%) and generally bears favorable prognosis in absence of *FLT3* mutations (present in about 40% of *NPM1*-mutated AMLs) (Suzuki et al., 2005).

NPM1 represents an ideal biomarker for AML as it is a very stable mutation. *NPM1* detection and quantification is currently used for MRD detection in *NPM1*-mutated AML (Gorello et al., 2006; Schnittger et al., 2009; Ivey et al., 2016). Ivey and colleagues have shown that persistent *NPM1* mutations after chemotherapy are associated with a greater risk of relapse after 3 years of follow up and with a lower OS (Ivey et al., 2016). *NPM1* mutations can be detected in most patients at the time of relapse (Ivey et al., 2016). However, a small subset of patients (~9%) loses NPM1 mutations at relapse (Krönke et al., 2013). In a large retrospective cohort of *NPM1*-mutated AML patients, it has been shown that other mutations may be detected by NGS in the BM of the patients achieving *NPM1* negativity after induction treatment, with a prognostic impact on progression free survival (PFS) and OS (Cappelli et al., 2022).

Standard therapy for *NPM1*-mutated patients includes 7 + 3 (daunorubicin and cytarabine) induction chemotherapy followed by consolidation. Given that *NPM1*-mutated AMLs often present high levels of CD33, the addition of gemtuzumab ozogamicin to conventional 7 + 3 chemotherapy may improve survival, but is currently under evaluation in clinical trials (NCT04168502). Moreover, *NPM1*-mutated AML cells are characterized by high expression of *HOXA* and *HOXB*, which are in turn controlled by histone modifiers Menin-*MLL1* and *DOT1L* (Brunetti et al., 2018; Kühn et al., 2016). Hence, the inhibition of these two hubs has shown synergistic activity both *in vitro* (Carter et al., 2021) and *in vivo* (Uckelmann et al., 2020). The use of Menin inhibitors as new therapeutic strategy in *NPM1*-mutated AML is being tested in clinical trials (#NCT04065399, #NCT04067336). In addition, the targeting of exportin 1 (XPO1), which down-modulates *HOX* expression maintained by *NPM1* mutation, is being investigated as antileukemic approach (Brunetti et al., 2018). So far, XPO1 inhibitors (first generation – Selinexor - and second generation – Eltanexor) have been combined with BCL2 inhibitor venetoclax and showed synergistic effect on AML cells *in vitro* (Luedtke et al., 2018). Eltanexor shows a more tolerable toxicity profile in AML patients compared to the first generation Selinexor

Table 3
Frequency and prognostic significance of recurrent gene mutations and newer agents for the targeted treatment of AML.[a].

Mutation	Frequency in AML	ELN risk category	Drugs	Drug Target	US FDA Approval
FLT3	ITD: 27–34%	ITD: intermediate	Midostaurin (TKD and ITD) Quizartinib (ITD)	Tyrosine kinase	April 2017 March 2022
	TKD: 11–12%	TKD: unclear	Crenolanib (TKD and ITD) Gilteritinib (TKD and ITD)		December 2017 November 2018
c-KIT	10–25%	Adverse	Imatinib Dasatinib	Tyrosine Kinase	None (Phase I/II clinical trial)[b,c,d] None (Phase II clinical trial)[e,f]
NRAS	11–30%	Intermediate	Tipifarnib	Farnesyl transferase	None
KRAS	15%	None	Antroquinolol	Farnesyl transferase	None (Phase II clinical trial- NCT03823352)
NPM1	30–50%	Favorable	Gemtuzumab-ozogamicin	CD33, MLL1, XPO1	September 1, 2017
CEBPA	15–19%	Favorable	None	N.A.[g]	N.A.[g]
RUNX1	5–15%	Adverse	None	N.A.[g]	N.A.[g]
DNMT3A	18–22%	None	None	N.A.[g]	N.A.[g]
IDH1	7–14%	Unclear	Ivosidenib	IDH1	July 2018
IDH2	8–19%	Unclear	Enasidenib	IDH2	August 2017
TET2	7.7–27.4%	None	None	N.A.[g]	N.A.[g]
ASXL1	5%	Adverse	None	N.A.[g]	N.A.[g]
WT1	10%	Adverse	None	N.A.[g]	N.A.[g]
TP53	5–20%	Adverse	APR-246	TP53 mutant protein	None (Phase II clinical trial- NCT03931291)

[a] Adapted from Padmakumar et al., 2021), (Padmakumar et al., 2021);
[b] (Advani et al., 2010);
[c] (Brandwein et al., 2011);
[d] (Heidel et al., 2007);
[e] (Marcucci et al., 2020);
[f] (Paschka et al., 2018);
[g] N.A.: Not Available.

(Etchin et al., 2017). However, further investigations are required to validate its efficacy in NPM1-mutated AML patients.

4.8. *FLT3* mutations

The FMS-like tyrosine kinase 3 (*FLT3*) gene mutations are among the most recurrent somatic mutations in cytogenetically normal AML (25–45% of cases) (Renneville et al., 2008). They result in the production of a constitutively active receptor tyrosine kinase (RTK) leading to leukemogenesis. *FLT3* is described as a late event in leukemogenesis, since other founding hits are required to determine overt AML. Two major types of *FLT3* mutations have been described in AML: the Internal Tandem Duplication (ITD), caused by the insertion of repeated nucleotides in the region belonging to the juxtamembrane (JM) domain, and point mutations occurring in nucleotides encoding for the activation loop of the Tyrosine Kinase Domain (TKD) (Grafone et al., 2012). *FLT3*-ITD predominantly occurs in young adults and in older patients (>60 years of age) (Schneider et al., 2012). The prognostic significance of *FLT3*-ITD allelic ratio (AR) quantification (Sakaguchi et al., 2020), was originally included in the old ELN 2017 risk classification of AML, considering mutant allele levels below a threshold of 0.5 associated to a favorable prognosis. The new ELN genetic risk classification does not longer consider the *FLT3*-ITD allelic *ratio* and incorporated AML with FLT3 mutations in the intermediate risk group, if concurrent to the presence of NPM1 mutation, and in the adverse group in the other cases. *FLT3*-TKD mutations are rarer and have a much less clear clinical impact on prognosis (Bacher et al., 2008), but provide a reliable biomarker for AML.

Nowadays, FLT3 represents a druggable target through specific inhibitors (Kiyoi et al., 2020), some of them already approved by Food and Drug Administration (FDA) and European Medicines Agency EMA (EMA). The first generation FLT3 inhibitors (midostaurin, sorafenib) were tested alone or in combination with conventional chemotherapy. Results from a randomized clinical trial showed that the addition of midostaurin to standard chemotherapy improves the OS in AML patients (Stone et al., 2017). The second generation FLT3 inhibitors (quizartinib, crenolanib and gilteritinib) are more specific and potent in monotherapy. Quizartinib, induced an improvement in the OS in relapsed/refractory *FLT3*-ITD + AML patients if compared to salvage chemotherapy (Erba et al., 2023). Similar results were obtained with gilteritinib (Gebru and Wang, 2020), inducing a higher OS in AML patients regardless of *FLT3* mutation type (ITD vs TKD); crenolanib in combination with chemotherapy induced higher rates of responses (72% of patients had CR) than chemotherapy alone.

FLT3-ITD is still one of the most important biomarkers in AML and its molecular testing is considered standard of care to determine the best therapeutic option both at diagnosis and relapse. This is epitomized by the notion that >70% of *FLT3*-ITD + AML patients at diagnosis present this mutation at relapse (Krönke et al., 2013).

Despite being reliably detectable and quantifiable as ITD (Grunwald et al., 2014; Lin et al., 2013; Schiller et al., 2012), *FLT3* is not a stable MRD marker, as its mutation levels can fluctuate over time. This phenomenon also depends on the treatment the patients receive: upon the administration of specific inhibitors *FLT3* levels can fall below the detectability threshold and they can suddenly rise in case of relapse.

4.9. *RAS* mutations

Mutations in members of the *RAS* oncogene family (*NRAS* and *KRAS*), encoding for GTPase proteins, are among the most frequent driver hits in AML. Their overall frequency in AML patients is of 15–40% (Liu et al., 2019), *NRAS* accounting for 11–30% of the cases (Bacher et al., 2006). The *RAS* signaling pathway has been found preferentially perturbed in certain AML subtypes (*e.g.* M4 (Bowen et al., 2005), where *NRAS* mutation is detectable at a frequency of 60–80% and *KRAS* at 20%). The impact of *RAS* mutations and their potential use as biomarkers is still debated (Liu et al., 2019; Bacher et al., 2006). However, in a large retrospective NGS analysis performed in AML patients, *RAS* mutations have been included in the group of mutations associated with Clonal Hematopoiesis of Oncogenic Potential (CHOP) and have shown to affect prognosis. Farnesyltransferase inhibitors (FTIs), which interfere with *RAS* function, have been tested in AMLs, but they showed modest efficacy and high rate of adverse effects due to the modulation of other targets, leading to side effects (Johnson et al., 2014).

4.10. *KIT* mutations

KIT gene encodes for a protein belonging to the type III RTK family (Paschka et al., 2006), which can be found highly upregulated or mutated in AMLs. Gain of function mutations, causing ligand-independent activation of KIT, have been described among all AML subtypes, but preferentially in the M2 group (Wang et al., 2015a). *KIT* mutations have a prognostic significance only in selected patients' cohorts, such as t(8;21) and inv(16) AMLs, where they confer a significantly worse outcome (Cairoli et al., 2006). Therefore, at present, *KIT* gene is not considered as a reliable prognostic biomarker for AML. No specific KIT inhibitors have been developed so far. However, several TKIs present activity against KIT, *e.g.*, imatinib (Doepfner et al., 2007), dasatinib (Schittenhelm et al., 2006), nilotinib (Malaise et al., 2009), with a mixed efficacy among different mutants.

4.11. *CEBPA aberrations*

The product of the CCAAT/enhancer-binding protein-alpha (*CEBPA*) gene acts as a key regulator of the proliferation and differentiation of myeloid precursors (Sarojam et al., 2015). Biallelic *CEBPA* mutations have been associated with a favorable outcome of AML patients (Fröhling et al., 2004; Wilhelmson and Porse, 2020), configuring a separate AML entity in the 2016 WHO classification and categorized in the favorable risk category with the newest ELN classification. However, a recent study investigating the prognostic impact of *CEBPA* mutations in 1028 AML patients, showed that in-frame monoallelic mutations affecting *CEBPA* basic leucine zipper (bZIP) region confer a favorable outcome, whereas *CEBPA* mutations out of the bZIP domain do not (Wakita et al., 2022). The favorable outcome of the in-frame bZIP *CEBPA* mutation is independent from mono- or bi-allelic mutational status. Interestingly, double-mutated *CEBPA* patients (91% of the cases) showed at least one bZIP mutation, whilst only 32% of the single-mutated *CEBPA* AML patients are mutated in the bZIP region. This finding holds potential for a refinement of the next AML risk classifications.

4.12. *RUNX1 aberrations*

The *RUNX1* gene, also known as *AML1*, encodes for a transcription factor that plays a crucial role in normal hematopoiesis. This gene is often affected by chromosomal translocations including t(8;21), t(3;21) and t(12;21), all involving the portion of chromosome 21 where it resides (Gaidzik et al., 2011; Saultz and Garzon, 2016). As described in the above sections, *RUNX1* has been associated with CBF-AML, a distinct entity with a favorable outcome. On the other hand, *RUNX1* point mutations are rare (Tang et al., 2009) and can be associated to the FAB M0 subtype, normal karyotype, and a distinct gene expression pattern upregulating several lymphoid genes (Gaidzik et al., 2011; Tang et al., 2009). *RUNX1* point mutations confer a very unfavorable outcome to AML (shorter OS and Relapse Free Survival-RFS) (Gaidzik et al., 2011; Greif et al., 2012), hence *RUNX1*-mutated AML are categorized in the adverse-risk group (Döhner et al., 2017).

4.13. *WT1 mutations*

Wilm's tumor 1 (*WT1*) gene is overexpressed in several leukemias,

including AML. *WT1* gene mutations are detectable in about 10% of AML patients and it determines an adverse prognosis at diagnosis (Renneville et al., 2008) and poor response to chemotherapy (Virappane et al., 2008). Hence, *WT1* represents an established biomarker for AML to be sequentially monitored in patients over their clinical course, since it has been shown to correlate with relapse risk (Malagola et al., 2016; Renneville et al., 2008). The sensitivity of *WT1* quantification in current assays is limited by the low expression levels of the wild-type gene in the normal population (Cilloni et al., 2009). To overcome this issue, combination of RQ-PCR with MFC has improved the predictive value of this biomarker (Marani et al., 2013).

4.14. IDH mutations

Mutations of *IDH1* and *IDH2* genes, respectively encoding dehydrogenase 1 and its mitochondrial homolog, lead to an aberrant production of the oncometabolite 2-hydroxyglutarate causing DNA methylation, toxic damage and genomic instability (Lu et al., 2012; Prada-Arismendy et al., 2017; Reitman and Yan, 2010). *IDH2* is more frequently mutated (8–19% of AML cases) (Montalban-Bravo and DiNardo, 2018), with different amino acidic substitutions based on different point mutations (R140 and R172, the commonest) and it is considered as an independent favorable prognostic factor (Chou et al., 2007). *IDH1* mutations are instead linked with an adverse outcome (OS and EFS) (Montalban-Bravo and DiNardo, 2018). *IDH* mutations appear to be good biomarkers for AML, MRD and relapse (Debarri et al., 2015). Two promising IDH inhibitors have currently entered the clinical arena, ivosidenib (IDH1) and enasidenib (IDH2). They both act as epigenetic modifiers inducing differentiation of blast cells. A phase II study has evaluated the effect of the addition of ivosidenib or enasidenib to induction standard chemotherapy, in relation to the patient's specific mutation, showing high response rates (93% for ivosidenib and 73% for enasidenib). The results of another phase III study indicated the promising activity of ivosidenib administered in combination with intensive chemotherapy, at consolidation and maintenance (#NCT03839771). Trials evaluating IDH inhibitors combination with conventional demethylating agents such as azacytidine (#NCT03683433 and #NCT03173248) are in progress (Donker and Ossenkoppele, 2020).

4.15. DNMT3A, TET2, ASXL1 (DTA) mutations

In recent years, efforts in the research of the mutation hierarchy of AML have identified *DNMT3A*, *TET2* and *ASXL1* genes (together referred as DTA genes), as the earliest culprits of genomic instability in HSCs. DTA mutations were found related to CHIP, which is not a tumor-defining condition per se, but a pre-leukemic state where HSCs cells are more prone to errors and can ultimately transform into leukemic cells if they acquire novel driver mutations. Therefore, DTA genes are useful indicators of CHIP and pre-leukemic BM instability, which cannot serve as direct biomarkers for AMLs. While *DNMT3A* and *TET2* do not have a specific impact on AML patients' outcome (Gaidzik et al., 2011), *ASXL1* mutations have been included in the adverse prognosis group of the most recent ELN classification, since they are often associated to myelodysplastic syndromes (MDS) and therapy-related AML (t-AML).

4.16. TP53 mutations

TP53 is the most frequently mutated gene in human cancers. It can be disrupted in AML patients, conferring an adverse prognosis also in this context (Sallman et al., 2020). In AML, *TP53* mutations occur in about 12% of AML patients (Grossmann et al., 2012), mainly in elderly previously exposed to intensive chemotherapy and with adverse karyotype (~70%) (Barbosa et al., 2019). Therapy-related/MDS-transformed AML also have a higher frequency of *TP53* mutations (Wong et al., 2015). Once detected, *TP53* can be used as a disease biomarker, since *TP53*-mutated clones tend to persist after conventional chemotherapy and can eventually be amplified by clonal selection. Specific therapeutic agents against mutant TP53 are traditionally scarce. One trial, using APR-246 as a single agent to reactivate the transcriptional activity of mutant TP53 and restore the wild-type function, has shown activity in AML patients (Asghari and Talati, 2020). Currently, regimens adopting demethylating agents (decitabine (Welch et al., 2016) or azacytidine), either alone or in combination with BCL2 inhibitor venetoclax, have shown some antileukemic activity also in *TP53*-mutated AML (Aldoss et al., 2018). APR-246 and azacytidine combinations are also under evaluation and are showing some clinical benefit (overall response rate of 75% and CR of 56% in a mixed cohort of high-risk MDS and AML) (Cluzeau et al., 2021).

5. Non-coding RNA as novel biomarkers in AML

The complexity of higher organisms mainly relies on the non-coding part of the genome, which represents around the 98% of the whole human transcriptome (Mattick, 2001). MicroRNAs (miRNAs) and long non-coding RNA (lncRNAs) affect key developmental hematopoietic programs, such as cell pluripotency, lineage specification and cell maturation/differentiation (Alvarez-Dominguez and Lodish, 2017; Bartel, 2009; Bissels et al., 2012). The choice of non-coding RNAs as novel source for future cancers' biomarkers is supported by the growing availability of deep sequencing data in the routine clinical practice, with increasingly cheap costs. Moreover, protein-coding RNAs can be perturbed by several regulatory pathways, often making the measured RNA levels diverging from the ultimate protein functions (Liu et al., 2016). Conversely, non-coding RNAs are already the functional form of the route; their expression can be associated more directly to the clinical outcome, when a clinically relevant non-coding RNA is individuated.

MiRNA is one of the earlier classes of non-coding RNAs inspected in relation with the onset and progression of AML (Wallace and O'Connell, 2017). They are evolutionarily conserved and induce post-transcriptional gene silencing through a limited base-pairing with complementary sequences in the 3'-untranslated regions (3'UTR) of target mRNAs (Bartel, 2009). The specific role of miRNAs in AML depends on the molecular events altering their expression levels or their mRNA targets. These events include chromosomal translocations (Bousquet et al., 2008), gene mutations (Trissal et al., 2018), copy number alterations (Ramsingh et al., 2013) and epigenetic deregulation of miRNA genes' transcription (Dzikiewicz-Krawczyk, 2014; Pagano et al., 2013; Zardo et al., 2012). In the perspective of finding new biomarkers for AML, miRNAs have been regarded as promising candidates. Specific signatures of miRNAs allow the differential diagnosis between Acute Lymphoblastic Leukemia (ALL) and AML, with an accuracy of 95% (Mi et al., 2007). In addition, they have been proposed for the classification of distinct AML subtypes and to predict their response to treatment. The screening of circulating miRNAs may be useful to detect de novo AML and for MRD monitoring (Wallace and O'Connell, 2017; Hornick et al., 2015; Zhi et al., 2013). Importantly, pre-clinical studies supported the therapeutic potential of miRNAs, such as miR-21, miR-29b, miR-126, miR-181a, miR-223 and miR-196b, showing encouraging results also in combination with standard chemotherapy (Wallace and O'Connell, 2017; Fletcher et al., 2022). These results were achieved overcoming issues as short half-life of synthetic miRNAs or inefficient delivery to the bone marrow (Wallace and O'Connell, 2017; Fletcher et al., 2022).

LncRNAs, generally defined as RNAs longer than 200 nucleotides without the canonical coding function of mRNAs, also affects gene expression (Ponting et al., 2009), and represent another source of potential disease biomarkers. A recognized function of lncRNAs is the sponging of miRNAs in response to specific cell stimuli (Salmena et al., 2011). LncRNA have already been tested as AML prognostic biomarkers with promising results (Pashaiefar et al., 2018; Yang et al., 2018), including lncRNA AML "signatures" (De Clara et al., 2017). However, lncRNAs can act by sequestering or releasing cluster of miRNAs (Xing

et al., 2015; Zhao and Liu, 2019), which adds a further layer of complexity to the evaluation of miRNA and lncRNAs levels as prognostic biomarkers in AMLs. In fact, despite the perspective of employing miRNAs and lncRNA as biomarkers for AMLs has already encountered the support of researchers (Loganathan and Doss, 2023), presently they are not used in clinical practice. The functions of noncoding RNAs in normal and malignant hematopoiesis and potential clinical implications have been extensively reviewed (Alvarez-Dominguez and Lodish, 2017; Wallace and O'Connell, 2017; Gourvest et al., 2019).

6. The epitranscriptome: clinical and therapeutic implications in AML

All RNAs (coding and noncoding RNAs) can be subjected to dynamic and reversible chemical modifications on RNA bases. These events elicit the realization of an "epitranscriptome", where RNA function are regulated by RNA modifications and the recruitment of effector RNA binding proteins, extending the concepts of epigenetics from DNA to RNA. Epitranscriptome represents an additional layer of gene expression regulation. Its deregulation in hematopoietic cells, in conjunction or not with chromosomal alterations and gene mutations, is now regarded as a mechanism leading to leukemia (Rosselló-Tortella et al., 2020). The measure of RNA transcript modifications, including pseudoridylation (Guzzi et al., 2018), adenosine to inosine (A-to-I) RNA editing (Crews et al., 2023; Xiao et al., 2019), RNA ribose 2'-*O*-methylation (Pauli et al., 2020; Zhou et al., 2017) and *N*6-methyladenosine (m^6A), which have been recently associated to AML pathogenesis (Barbieri et al., 2017; Vu et al., 2017; Weng et al., 2018), or the expression levels of the RNA binding proteins and enzymes catalyzing them, represents a promising practice for assessing prognosis and improving treatment for AMLs. The upgrade of multiple techniques, allowing the study of the epitranscriptome, opened new perspectives in this field.

6.1. Pseudoridylation (Ψ)

Pseudoridylation is a post-transcriptional isomerization reaction that converts a uridine to a pseudouridine (indicated by the symbol "Ψ") within an RNA chain, catalyzed by a family of Pseudouridine Synthase (*PUSs*) (Ge and Yu, 2013). Ψ is the most abundant modified nucleotide in different classes of RNA and can been mapped through various methods based on its labeling with N-cyclohexyl-N′-b-(4-methylmorpholinium) ethylcarbodiimide (CMC) (Carlile et al., 2014; Li et al., 2015). Recent evidence in human embryonic stem cell lines (hESCs), indicates that the knockout of the pseudouridine synthase 7 (*PUS7*) impairs HSCs differentiation through the abrogation of pseudouridylation (Ψ) on a class of RNA fragments derived from transfer RNA (tRFs) that contain a 5' terminal oligoguanine (TOG), named "mini-TOG" (mTOG), due to their short length. The abrogation of Ψ on mTOG decrease their levels. Interestingly, reduced levels of *PUS7* and mTOG, associated to an aberrant rate of overall protein synthesis, were detected in HSCs from MDS and AML patients (Guzzi et al., 2018).

6.2. A-to-I editing

Adenosine-to-Inosine (A-to-I) editing recalls other cases of AML-related epitranscriptome alterations. A-to-I editing is catalyzed by the enzyme Adenosine Deaminase Acting on RNA 1 (*ADAR1*), which converts A-to-I on double-strand RNAs target, and the resulting inosine shares base-pairing properties with guanosine (Bass et al., 1997; Wang et al., 2000). A-to-I editing is usually identified by RNA-sequencing, since inosine bases are read as guanosine rather than adenosine residues (Pinto and Levanon, 2019). However, this method can lead to misinterpretation due to single-nucleotide polymorphisms or point mutations (Nguyen et al., 2022). The effects of deregulated A-to-I editing activity were initially reported in CD34+CD117+ AML blasts, where it generated splicing variants of tyrosine phosphatase non-receptor type 6 (*PTPN6*) mRNA, encoding for a SH2 phosphatase modulating myeloid cell signaling (Beghini et al., 2000). Increased ADAR1 levels were measured in AML patients' BM mononuclear cells, where it promotes leukemogenesis through the Wnt pathway (Xiao et al., 2019). Importantly, Rebecsinib (17S-FD-895), an inhibitor of splicing-mediated ADAR1 activation, at doses that spare normal HSCs, prevents the increased expression of *ADAR1p150*, a splicing variant of *ADAR1*, which induces HSCs (CD34+CD38−Lin−) and HPCs (CD34+CD38+Lin−) into self-renewing LSCs, driving secondary acute myeloid leukemia (sAML) and therapeutic resistance (Crews et al., 2023).

6.3. 2'O-methylation

The methylation of ribose at 2'-OH group (2'O-methylation) is a prevalent RNA modification interesting the ribose of different types of RNAs, *e.g.*, transfer RNA (tRNA), ribosomal RNA (rRNA), small nucleolar RNA (snoRNA) and mRNA. It can occur on each RNA nucleotide and influences translation efficiency and other processes (Ayadi et al., 2019). The identification of 2'O-methylation relies on RNA resistance to alkaline hydrolysis determined by ribose methylation (Motorin and Marchand, 2018). However, the techniques identifying these RNA modifications will likely receive an enormous refinement from the recent advances in nanopore sequencing (Nguyen et al., 2022; Begik et al., 2021). Experimental evidences suggest that RNA 2'-O methylation contribute to the pathogenesis of *AML1-ETO*-AMLs. The enhanced self-renewal capabilities of *AML1-ETO* + blasts are associated to increased levels of Amino-terminal Enhancer of Split (AES), a transcription factor promoting leukemogenesis through the activation of the Wnt pathway (Steffen et al., 2011). The enhanced expression of AES increases the levels of C/D box snoRNAs (SNORDs), an abundantly expressed class of short non-coding RNAs, increasing the O-methylation of rRNAs. This ultimately enhances the overall protein synthesis, favoring LSC self-renewal (Zhou et al., 2017). The specific 2-O' methylation of 18S, mediated by SNORD42, is required for leukemic growth (Pauli et al., 2020). In fact, AML1-ETO transduced into AES-deficient mouse fetal liver cells fails to generate leukemia in mice (Zhou et al., 2017).

6.4. m^6A

*N*6-methyladenosine (m^6A) is the most abundant and conserved post-transcriptional chemical modification of eukaryotic mRNAs and long non-coding RNAs, playing important roles in the control of gene expression. m^6A modifications on mRNAs are now regarded as promising clinically relevant AML biomarkers and are furnishing unexpected cues for AML treatment. m^6A is mainly detected at the whole-transcriptome level using m^6A antibody affinity enrichment combined with high-throughput sequencing methods (MeRIP-seq or m^6A-seq, m^6A-seq2), and single-nucleotide mapping of m^6A (miCLIP-seq) (Dominissini et al., 2012; Linder et al., 2015; Dierks et al., 2021). These methods allowed a boost of data collection and individuated specific classes of mRNAs typically decorated by m^6A and frequently related to cell development or lineage cell fate, whereas those not affected by methylation are often housekeeping mRNAs (Dominissini et al., 2012; Linder et al., 2015; Geula et al., 2015; Meyer et al., 2012). Sequence analyses showed that m^6A peaks occur within the consensus motif DRACH (D = A/G/U; R = G/A; H = A/C/U), and are enriched near stop codon and around the proximal regions of 3'UTR, whose alterations can contribute to tumorigenesis (Dominissini et al., 2012; Meyer et al., 2012; Mayr and Bartel, 2009; Mayr, 2016; Quattrocchi et al., 2020; Wei et al., 2022). S-adenosy-L-methionine (SAM), an essential metabolite, serves as the high-energy methyl donor for this mRNA modification (Wang et al., 2016a).

Methylation events on RNA are dynamic and reversible post-transcriptionally regulated epigenetic effects important for proper gene regulation. m^6A modifications are mostly installed, recognized and

erased by m^{6}A methyltransferases ("writers"), m^{6}A-specific binding proteins ("readers") and demethylases ("erasers"), respectively, all mediating the recruitment of downstream functional protein complexes (Zaccara et al., 2019). Notably, normal hematopoiesis is governed by m^{6}A modifications on mRNA (Vu et al., 2017; Cheng et al., 2019). The expression levels or activities of m^{6}A regulators have been found dysregulated in AMLs, thus providing new targets for AML diagnosis and treatment (Vu et al., 2017; Yao et al., 2018).

The m^{6}A methyltransferases complex (m^{6}A MTase, "writers"), installing the m^{6}A marks, includes different subunits. The methyltransferase-like 3 (METTL3) is the SAM core catalytic subunit, transferring the methyl group to the *N*6 of the adenosine residue. This enzymatic reaction occurs co-transcriptionally within the nucleus, where the m^{6}A MTase complex is localized (Huang et al., 2019a). METTL3 operates as a heterodimer with methyltransferase-like 14 (METTL14), stabilizing the complex, facilitating RNA recognition and binding (Wang et al., 2016a, 2016b; Śledź and Jinek, 2016). For full mRNA targeting activity and intracellular localization, the METTL3-METTL14 complex, also known as m^{6}A-METTL Complex (MAC), requires other associated proteins, forming the m^{6}A-METTL Associated Complex (MACOM). MACOM complex includes: the crucial adaptor Wilms tumor 1 associated protein (WTAP), facilitating the localization of MAC complex to chromatin (Schöller et al., 2018); Vir-like m^{6}A methyltransferase associated protein (VIRMA), interacting with WTAP; WTAP is required for m^{6}A mRNA methylation near stop codon and in 3'UTR (Schwartz et al., 2014), and is involved in transcript polyadenilation (Yue et al., 2018); RNA-binding motif protein 15/15B (RBM15/15B), whose binding to WTAP localize the complex at specific RNA sequences; Zinc finger CCHC-type containing 13 (ZCH13), interacting with WTAP and RBM15/15b and assisting the nuclear localization of the complex (Knuckles et al., 2018; Wen et al., 2018); HAKAI, also known as Cbl proto-oncogene like 1 (*CBLL1*), is an E3 ubiquitin-ligase that associates with MACOM to maintain m^{6}A levels (Horiuchi et al., 2013). A schematic representation of the components of MACOM complex is shown in Fig. 1a.

Following methylation within the nucleus, the m^{6}A-modified mRNA is recognized and bound by m^{6}A-specific binding proteins (m^{6}A "readers"), which includes the YTH-domain family proteins YTH N6-methyladenosine RNA binding protein 1/2/3 (YTHDF1, YTHDF2, YTHDF3) and YTH Domain-Containing 1/2 (YTHDC1 and YTHDC2), controlling mRNA maturation, translation and decay (Zaccara et al., 2019). The m^{6}A reader YTHDF1 binds the eukaryotic initiation factor 3 (eIF3) and enhances the initiation of translation (Wang et al., 2015b). Interestingly, translation efficiency can be modulated directly by eIF3 recruited at m^{6}A sites on 5'UTR of the RNA transcript, without the binding to m^{6}A readers (Meyer et al., 2015), or through eIF3 binding to the METTL3 subunit, inducing mRNA circularization, which facilitates the ribosome recycling (Choe et al., 2018).

YTHDF2 induces the degradation of m^{6}A-containing targeted mRNAs (Wang et al., 2014). However, for the majority of mRNAs affected in stability by m^{6}A, other unknown factors are likely to be involved (Ke et al., 2017). YTHDF3 associates with both YTHDF1 and YTHDF2, assisting their functions on mRNA (Meyer and Jaffrey, 2017).

The m^{6}A methylation has been implied in other cellular processes, such as mRNA splicing, even if circumscribed to a limited number of transcripts (Ke et al., 2017), and mRNA nuclear export (Roundtree et al., 2017), although these evidences are still debated. Moreover, mRNAs with multiple m^{6}A sites could act as a platform to mediate the interactions between YTHDF proteins in the cytoplasm, leading to the peculiar subcellular compartmentalization known as liquid-liquid phase separation (LLPS). LLPS sorts mRNAs and proteins and organizes their localization into biomolecular condensates such as stress granules, P-bodies and neuronal RNA granules, influencing mRNA stability or translation (Ries et al., 2019).

In contrast to the YTHDF proteins, YTHDC1 is predominantly localized into the nucleus, where it appears to be the major m^{6}A reader

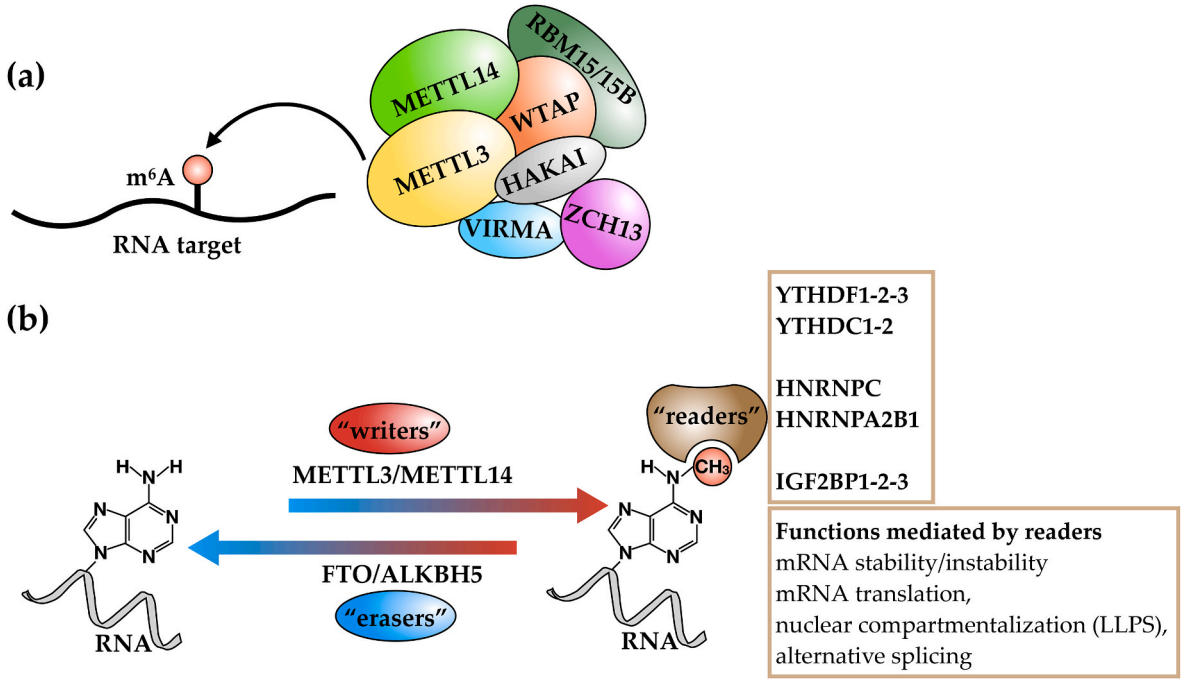

Fig. 1. Schematic representation of the complex deposing m^{6}A on RNA and role of m^{6}A-related enzymes acting as writers, erasers and readers. (a) The heterodimeric complex formed by METTL3 and METTL14 (Wang et al., 2016a, 2016b; Śledź and Jinek, 2016), with the cooperation of other essential cofactors such WTAP, HAKAI, ZCH13, VIRMA and RBM15/15B (Schöller et al., 2018; Schwartz et al., 2014; Yue et al., 2018; Knuckles et al., 2018; Wen et al., 2018; Horiuchi et al., 2013), is responsible for the catalytic reaction deposing the methyl group on RNA adenosine residues. **(b)** The balance of m^{6}A on RNA is determined by writers METTL3/METTL14 and erasers FTO/ALKBH5 (Jia et al., 2011; Mauer et al., 2019; Zheng et al., 2013). m^{6}A residues are recognized by readers, mediating various and in some case opposite biological functions, highly context-dependent (Wang et al., 2014, 2015b; Meyer et al., 2015; Choe et al., 2018; Ke et al., 2017; Meyer and Jaffrey, 2017; Roundtree et al., 2017; Ries et al., 2019; Cheng et al., 2021; Liu et al., 2015; Alarcón et al., 2015; Huang et al., 2018).

(Meyer and Jaffrey, 2017). In addition, YTHDC1 mediates nuclear LLPS, forming nuclear YTHDC1-m^{6}A condensates (nYACs) (Cheng et al., 2021). These nuclear compartments are enriched in AML cells and can promote leukemia by protecting from degradation m^{6}A-transcripts related to proliferation and survival, such as the c-MYC proto-oncogene mRNA (Cheng et al., 2021). In addition, Heterogeneus Nuclear Ribonucleoprotein C (HNRNPC), involved in structure-dependent accessibility of RNA (Liu et al., 2015), Heterogeneus Nuclear Ribonucleoprotein A2B1 (HNRNPA2B1), affecting alternative splicing and miRNA processing (Alarcón et al., 2015), and Insulin-Growth Factor 2 mRNA-binding proteins (IGF2BPs), promoting RNA and translation stability, act also as m^{6}A readers (Huang et al., 2018).

Beside "writers" and "readers", the canonical scheme of epitranscriptomic modulation, includes also the "erasers", the enzymes that remove the modification (Fig. 1b). For m^{6}A, at the present time only two erasers are known. The first to be identified is fat-mass- and obesity-associated protein (FTO) (Jia et al., 2011). However, the specificity of this enzyme toward m^{6}A has been questioned in subsequent works, claiming that FTO demethylating activity is directed mostly toward another RNA modification, 2-*O*-dimethyladenosine (m^{6}Am) (Mauer et al., 2019). Nevertheless, more recent evidences reported that FTO might have an active role in m^{6}A demethylation specifically in pathological contexts, as discussed later. The other known m^{6}A demethylase is AlkB Homolog 5 (ALKBH5) (Zheng et al., 2013); its knockout and overexpression significantly alter m^{6}A levels, indicating a more specific activity than FTO (Zaccara et al., 2019). In the physiological contexts, *ALKBH5* is required for mice spermatogenesis (Zheng et al., 2013), and plays a role also in the pathogenesis of neoplastic diseases, such as glioblastoma (Zhang et al., 2017a), breast cancer (Thalhammer et al., 2011) and AMLs, as described in the section 8.

7. m^{6}A RNA modifications in normal hematopoiesis

Recent findings strongly support the involvement of m^{6}A RNA modifications in the process that defines the genesis of HSCs in the aorta-gonad-mesonephros (AGM), named endothelial-to-hematopoietic transition, and during the maturation/differentiation of hematopoietic cells (Lv et al., 2018; Zhang et al., 2017b). Accordingly, the silencing of the m^{6}A methyltransferase mettl3 in zebrafish embryos significantly decreases m^{6}A levels and impairs HSC generation (Zhang et al., 2017b). Further analysis performed in *mettl3*-silenced zebrafish cells, combining high-throughput MeRIP–seq and miCLIP–seq with RNA-sequencing, and selecting genes with significantly decreased m^{6}A levels and upregulated expression levels, individuated notch receptor 1a (*notch1a*) mRNA as a direct target of Mettl3. Mettl3-induced m^{6}A modifications delays the *notch1a* mRNA decay mediated by the m^{6}A reader Ythdf2, thus affecting HSC generation (Zhang et al., 2017b). Similarly, in mouse embryos, decreased *Mettl3* mRNA and protein levels in endothelial cells from the AGM region impairs HSC development and definitive hematopoiesis by reducing m^{6}A modifications and upregulating *Notch1* and signaling network (Lv et al., 2018). Importantly, the relevant role of *METTL3* expression during early embryogenesis is indicated by the lethal consequences of its knockout either in zebrafish or mouse embryos, compelling the use of inducible models to turn off *METTL3* expression in specific tissues and at defined times of embryogenesis (Lv et al., 2018; Zhang et al., 2017b).

Mettl3 loss-of-function studies in mice enforced the strength of the role of m^{6}A modifications in the regulation of adult hematopoiesis, particularly through the m^{6}A methylation of *Myc* transcript, a recognized downstream target of Mettl3 activity (Cheng et al., 2019; Lee et al., 2019). Conditional depletion of *Mettl3* in mice compromises HSC differentiation and whole blood cell distribution (Lee et al., 2019). *Mettl3*-deficient mice preferentially accumulate HSCs over committed progenitors, indicating that m^{6}A modifications are required at earlier stages of differentiation. Importantly, *Mettl3*-depletion in HSCs, but not in myeloid progenitors, affects myelopoiesis in mouse. Moreover, the analysis of m^{6}A-methylome in HSCs demonstrated that genes related to hematopoiesis are enriched in m^{6}A marks. *Myc*-m^{6}A methylation activates its expression, favoring the differentiation stages from HSCs to myeloid progenitors (Lee et al., 2019). Moreover, *Mettl3*-conditional knockout in mice, demonstrated that an m^{6}A-dependent gradient of *Myc* expression influences the symmetric commitment and identity of self-renewing HSCs, and results in an accumulation of HSCs with impaired differentiation, a feature that recalls AMLs (Cheng et al., 2019). Such data were confirmed in other *Mettl3* and/or *Mettl14* conditional knockouts mice models (Yao et al., 2018). However, the relationship between *METTL3* activity/expression and AML onset cannot be trivially derived, since it appears strictly dependent on the stage of the hematopoietic routes affected by m^{6}A alterations. For instance, the effects of the depletion of *METTL3* in hematopoietic cells at advanced stages of maturation/differentiation may be restricted to specific differentiation routes. In the human HEL cell line, a surrogate model of erythropoiesis, a whole-genome CRISRP–Cas9-based screening approach revealed a key regulatory role for m^{6}A RNA methylation during erythropoiesis. *METTL3*, *METTL14* and *WTAP*, all components of the m^{6}A MTase complex, appear required for maintaining the expression glycophorin (CD235a), a typical marker of erythroid differentiation, in HEL cells and for erythroid lineage specification in human HSPCs. Moreover, this study showed that m^{6}A modifications promotes gene expression programs through the translation of hundreds of mRNAs involved in erythropoiesis and erythroid-associated diseases or coding for histone methyltransferases and ribosome/RNA binding proteins (Kuppers et al., 2019).

8. m^{6}A RNA modifications in AML

Since m^{6}A modifications are tightly related to HSCs development, maturation and differentiation, it is not surprising that alteration of m^{6}A profiles and m^{6}A regulators expression levels have been detected and proposed as useful tool for MRD monitoring and targeted treatment in AMLs. Interestingly, m^{6}A levels in CD34$^+$ cells from AML patients are higher than human healthy donors' cord blood (CB)-derived CD34$^+$ HSC (Vu et al., 2017).

METTL3 mRNA and protein levels also are increased in AML blasts and leukemic cell lines if compared with CB-CD34$^+$ HSCs (Vu et al., 2017). Functional studies revealed that *METTL3* overexpression in human CB-CD34$^+$ HSCs promoted cell growth and inhibited cell differentiation, whereas its depletion enhanced myeloid differentiation (Vu et al., 2017). *METTL3* depletion in AML CD34$^+$ cells reduced m^{6}A levels, inhibited colony formation and induced blasts differentiation and apoptosis. Similar effects were detected following *METTL3* depletion in AML cell lines, delaying their leukemogenic activity in recipient mice (Vu et al., 2017). In these mechanisms, METTL3 controls m^{6}A levels and expression of targets *MYC*, *PTEN* and *BCL2*, whose increased expression sustains AML (Vu et al., 2017). In a mouse model of AML induced by *KMT2A-MLLT3* (or *MLL–AF9*) *FLT3*-ITD, *Mettl3* emerged from a CRISPR-Cas9 screening as a gene whose expression is required for ex-vivo growth of mouse primary AML cells (Barbieri et al., 2017). METTL3 and METTL14 localize at the transcriptional start sites of genes enriched by H3K4me3 marks and the CAATT-box binding protein CEBPZ, which recruits METTL3 to chromatin (Barbieri et al., 2017). The METTL3/CEBPZ-dependent m^{6}A modifications can occur within the coding sequence of a subgroup of mRNAs and, in turn, control their translation without affecting the transcripts levels. This subgroup includes the transcription factors *SP1* and *SP2* mRNAs (Barbieri et al., 2017), whose enhanced levels induced by promoter-bound METTL3/-CEBPZ increases *MYC* expression and AML cell growth (Barbieri et al., 2017).

Recently, the expression of METTL3 was related to AML treatment outcome and chemoresistance via a METTL3-m^{6}A- Integrin Subunit alpha 4 (ITGA4)-homing/engraftment axis. The METTL3 enhanced AML homing/engraftment in mouse models of AML can be reversed by

METTL3 inhibitor STM2457, described below, suggesting the potential efficacy of this drug in the treatment of refractory/relapsed AML (Li et al., 2022).

Similarly to *METTL3*, *METTL14* expression levels have been associated with the activation of a *SP1/MYB/MYC* circuitry and leukemogenesis (Weng et al., 2018). In mice BM cells, the decreased expression of *METTL14* during normal myelopoiesis is related to a reduction of total m^{6}A levels (Weng et al., 2018). Moreover, *METTL14* silencing in human CB CD34$^+$ HSC enhances monocyte/macrophage differentiation. However, mononuclear cells from the BM of AML patients carrying the t (8;21), t(15;17), and t(11q23) chromosomal abnormalities, express significantly higher *METTL14* levels than healthy donors' BM cells. Interestingly, in the APL cell line NB4, *METTL14* and m^{6}A levels are decreased upon treatment with differentiating agents such all-trans-Retinoic Acid (ATRA) or phorbol-12-myristate-13- acetate (PMA) (Weng et al., 2018). The induction of leukemia in mice leads to an increase of *Mettl14* levels (Weng et al., 2018), whereas *Mettl14* depletion impairs leukemogenesis in mice. A scheme resuming the reported roles of m^{6}A/METTL3/METTL14 upon myeloid differentiation and AML is shown in Fig. 2.

The overexpression of *WTAP*, another subunit of the MACOM complex, was related to AML proliferation, through a METTL3-dependent mechanism (Sorci et al., 2018). An increased expression of m^{6}A demethylases, the "erasers" that remove m^{6}A, also has been measured in different AML subtypes. The m^{6}A-demethylase *FTO* (Jia et al., 2011) is highly expressed in AMLs with genetic abnormalities, including t (11q23)/MLL, t(15;17)/*PML-RARA*, *FLT3*-ITD, and/or *NPM1* mutations, (Li et al., 2017). FTO sustained leukemogenesis by depleting m^{6}A levels in the downstream mRNA targets *ABS2* and *RARA*, decreasing their mRNA stability and protein levels (Li et al., 2017). Such as *METTL14*, whose expression level is a readout of blasts differentiation (Weng et al., 2018), the *FTO* overexpression has been found associated to resistance to ATRA differentiation therapy in AML (Li et al., 2017), further underling the functional relevance of m^{6}A methylation in leukemogenesis and response to treatment.

R-2-hydroxyglutarate (R-2HG), an endogenous metabolite produced in excess in *IDH1/2*-mutant AMLs, inhibits the function of the m^{6}A demethylase *FTO*, exerting an anti-leukemic activity in R-2HG-sensitive leukemia cells. By inhibiting FTO, R-2HG increases global m^{6}A levels, decreases the stability of *CEBPA* and *MYC* target transcripts, promoting cell-cycle arrest and apoptosis (Su et al., 2018). Accordingly, treatment with FB23-2, a small molecule acting as a specifical inhibitor of FTO activity, significantly reduces cell proliferation and induces differentiation/apoptosis in primary AML blasts and AML cell lines *in vitro* and *in vivo*, supporting its therapeutical potential in AMLs (Huang et al., 2019b).

The other m^{6}A demethylase *ALKBH5* also is overexpressed in AML blasts (Zheng et al., 2013). Its activity is required for LSC maintenance and is dispensable for normal hematopoiesis (Wang et al., 2020). The leukemogenic role of *ALKBH5* has been related to the m^{6}A-dependent increase stability of the receptor tyrosine kinase *AXL* mRNA (Wang et al., 2020). Another study related *ALKBH5* overexpression in AML to a positive regulation of the transforming Acidic Coiled-Coil Containing Protein 3 (*TACC3*), sustaining leukemia and found associated with a poor prognosis (Shen et al., 2020). Moreover, mutations or copy number variations of genes encoding for m^{6}A-regulators (*METTL3*, *METTL14*, *YTHDF1*, *YTHDF2*, *FTO*, and *ALKBH5*), have been associated to TP53 mutations, suggesting their complementary contribution to the onset and/or maintenance of AMLs (Kwok et al., 2017).

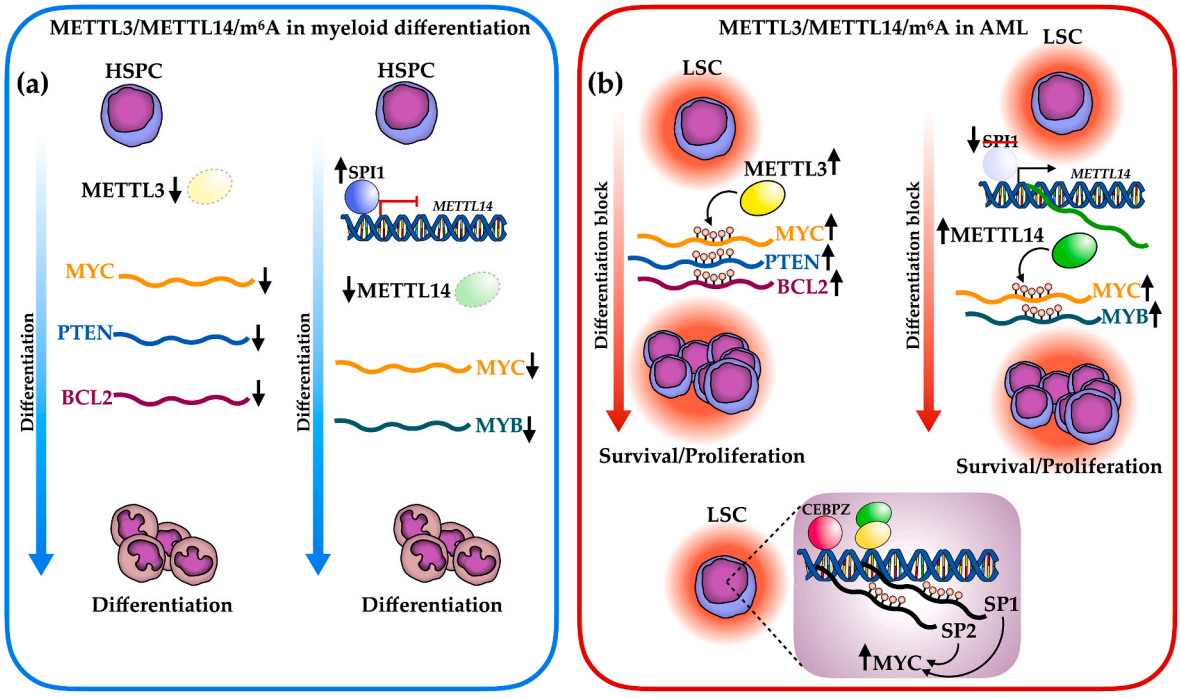

Fig. 2. Roles of METTL3/METTL14/m^{6}A in normal differentiation and in AML. General scheme of different mechanisms described in the text, related to the involvement of m^{6}A in normal myelopoiesis or in AML onset and maintenance. **(a)** Left: the expression levels of *METTL3* are reduced in HSPC undergoing myeloid differentiation, decreasing m^{6}A levels and expression of targets *MYC*, *PTEN* and *BCL2* (Vu et al., 2017). Right: in HSPC, the transcription factor SPI1 inhibits *METTL14* expression while myeloid differentiation proceeds, reducing m^{6}A levels and expression of *MYB* and *MYC* (Weng et al., 2018). **(b)** Left: in LSC *METTL3* expression is increased, causing the maintenance of *MYC*, *PTEN* and *BCL2* methylation, leading to differentiation block and leukemic state (Vu et al., 2017). Right: in METTL14-dependend AML, SPI1 activity in LSC is suppressed and *METTL14* expression is not turned off, leading to increased methylation of *MYC* and *MYB*, and resulting in blast survival and proliferation (upper right scheme) (Weng et al., 2018). Bottom: chromatin-dependent mechanism of METTL3/METTL14 activity in AML (Barbieri et al., 2017). In this case, METTL3, in cooperation with CEBPZ, binds the promoter region of several genes, including *SP1* and *SP2*, inducing their co-trascriptional methylation, ultimately resulting in MYC augmented expression (Barbieri et al., 2017). Abbreviations: HSPCs, Human Stem and Progenitor cells, LSC, Leukemic Stem Cells.

9. Therapeutic targeting of m^{6}A modifications in AML

The overexpression of the components of the m^{6}A MTase "writer" complex in AMLs pushed the research of the field to rapidly develop specific inhibitors that could affect the leukemogenic process, sparing normal hematopoiesis. Actually, inhibitors of the METTL3-mediated m^{6}A modifications are opening unexpected perspectives of therapeutic innovation in refractory/relapsed AMLs and possible implications for risk classification.

9.1. Structural aspects of the m^{6}A MTase "writer" complex

In the METTL3-METTL14 (MAC) subcomplex, METTL14 anchors the RNA substrate through the Arg-Gly-Gly sequence located at the C-terminus and recognizes the histone H3K36me3 to facilitate the positioning of the m^{6}A MTase complex near RNA polymerase II (Huang et al., 2019a). In this way, the m^{6}A MTase complex encounters new RNA transcripts, leading to the co-transcriptional methylation of *N*6-adenosine (Huang et al., 2019a). METTL3, instead, catalyzes the transfer of a methyl group from the co-substrate SAM to the target adenosine by means of a SAM-binding region within its methyltransferase domain (MTD) (Wang et al., 2016b; Liu et al., 2014). As previously mentioned, METTL14, although showing a MTD, does not possess catalytic activity, because it does not contain the SAM binding cleft (Wang et al., 2016b). Therefore, METTL14 acts as a scaffold for the interaction between METTL3 and the RNA substrate and allows the catalytic activity of METTL3, which is negligible in the absence of METTL14 (Wang et al., 2016a, 2016b; Fiorentino et al., 2023). The METTL3-METTL14 complex shows a butterfly shape (Fig. 3). METTL3 and METTL14 interact asymmetrically and in an antiparallel way through their methyltransferase domains (Fig. 3a) (Wang et al., 2016b).

The methyltransferase domain of METTL3 is characterized by eight β-sheets flanked by four α-helices and three 3_{10} helices. The SAM molecule forms several hydrogen bonds with the highly conserved Asp-Pro-Pro-Trp motif of MTD. In particular: (*i*) the Asp377 side chain and the main chain of Ile378 recognize the adenine moiety of SAM; (*ii*) Asp395, Lys513, His538 and Asn539 residues interact with the methionine portion of SAM; (*iii*) a conserved water molecule creates a contact bridge between Glu532 and Leu533 (Fig. 3) (Wang et al., 2016a); and (*iv*) Gln550, Asn548, and Arg536 residues establish hydrogen bonds with the hydroxyl groups of ribose (Fig. 3b) (Wang et al., 2016b). Moreover, the *N*-terminus of METTL3 binds the WTAP factor enabling the formation of the WTAP-METTL3-METTL14 complex (Śledź and Jinek, 2016; Schöller et al., 2018).

In the MACOM complex, the homodimeric binding of WTAP to METTL3 is crucial for the m^{6}A MTase complex formation (Su et al., 2022). WTAP does not possess methyltransferase activity but facilitates the deposition of m^{6}A by the MTase complex and is required for the localization of METTL3-METTL14 in the nuclear speckles (Meyer and Jaffrey, 2017; Liu et al., 2014; Fiorentino et al., 2023; Ping et al., 2014). VIRMA represents the largest component and acts as a bridge between the different subunits promoting the recruitment of MTase complex to specific m^{6}A methylation sites through its interaction with WTAP. Moreover, VIRMA plays a crucial role in the SAM-dependent methyltransferase activity; in fact, its depletion leads to a reduction in m^{6}A levels as a consequence of restricted access of the METTL3-METTL14 complex to the target mRNA (Garcias Morales and Reyes, 2021). The adaptor protein RBM15 allows the engagement of m^{6}A MTase complex on pre-mRNA (Schwartz et al., 2014), mediating its recruitment to U-rich RNA sites (Balacco and Soller, 2019). Moreover, RBM15 promotes ZC3H13 binding to WTAP and modulates the nuclear localization of m^{6}A MTase complex (Knuckles et al., 2018). Lastly, HAKAI, bound to WTAP and ZC3H13, displays E3 ubiquitin ligase activity (Su et al., 2022).

9.2. Inhibitor recognition by METTL3

Prototypical METTL3 inhibitors initially derived from the co-substrate SAM (Fig. 4) (Moroz-Omori et al., 2021). The Caflisch team, starting from crystallography studies, obtained the UZH1a (R-2a) compound, which exhibits a good inhibitory activity against METTL3 but possesses unfavorable absorption, distribution, metabolism, and elimination properties (Moroz-Omori et al., 2021). Additional modifications, such as the introduction of two fluorine atoms on the benzene core (*e.g.*, UZH2 (2p) compound), increased the IC_{50} from the micromolar to the nanomolar value (Table S1) (Dolbois et al., 2021).

To date, the most advanced METTL3 inhibitor is STM2457 (3b) (Yankova et al., 2021), showing efficacy in the treatment of various AML

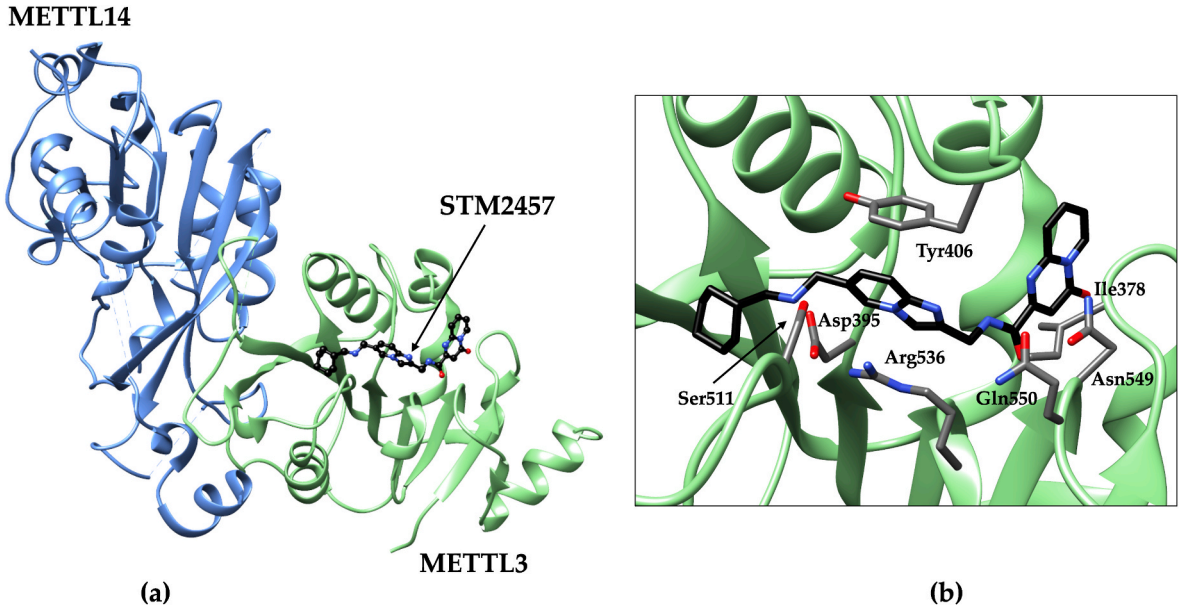

Fig. 3. Representation of METTL3-METTL14 dimer and key residues of METTL3 recognizing the competitive inhibitor STM2457. (**a**) Structure of STM2457 bound to the METTL3-METTL14 dimer (PDB ID: 7O2I) (Yankova et al., 2021). (**b**) Key residues of METTL3 recognizing the competitive inhibitor STM2457 are labeled. Pictures have been drawn by UCSF-Chimera (Pettersen et al., 2004).

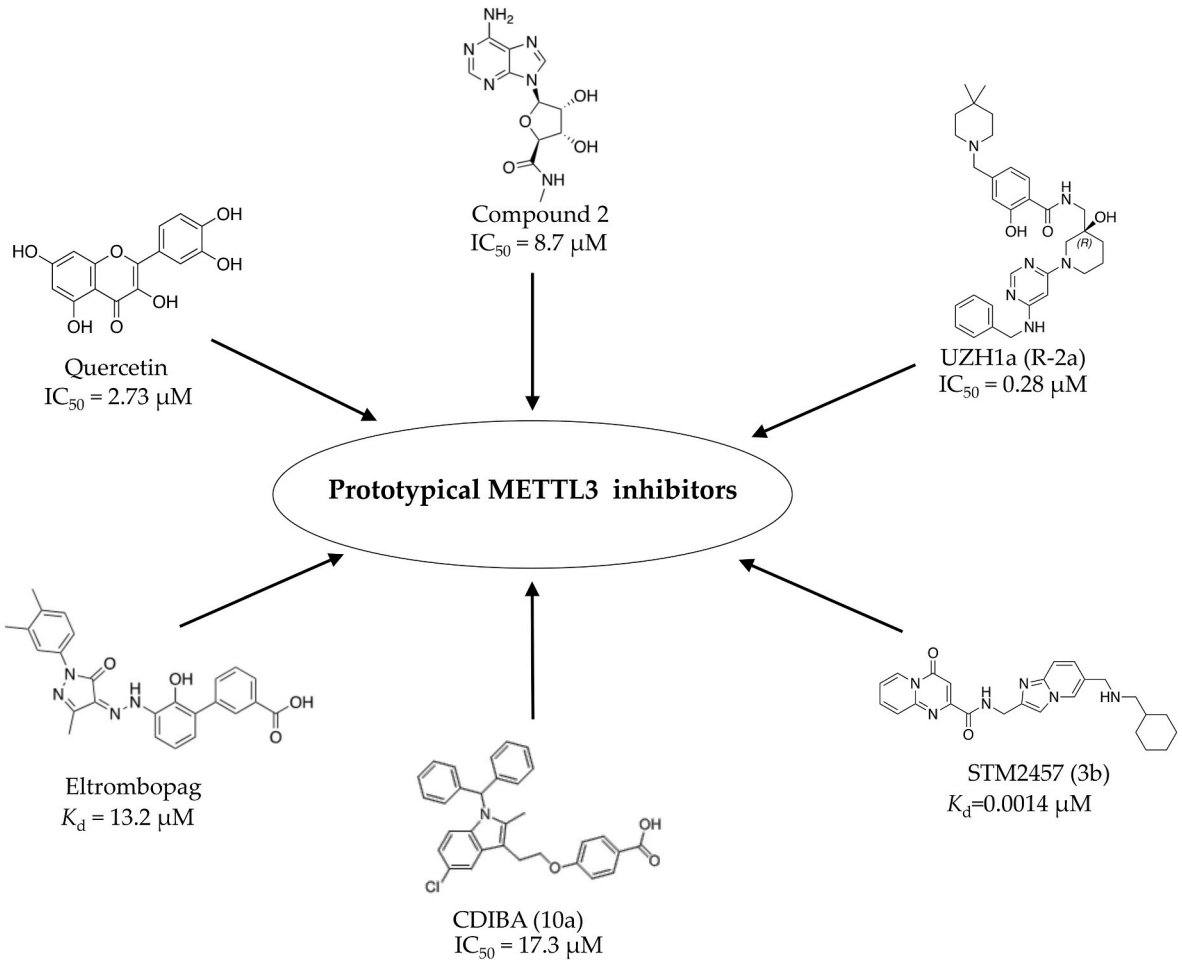

Fig. 4. Prototypical METTL3 inhibitors. (Roth et al., 2012); (Bedi et al., 2020); (Moroz-Omori et al., 2021); (Yankova et al., 2021); (Lee et al., 2022); (Du et al., 2022).

models. STM2457 (3b) binds competitively to the SAM binding pocket. In detail, the central amide of STM2457 (3b) forms two hydrogen bonds with Asn549 and a conserved water molecule, while the pyridopyrimidone carbonyl forms a hydrogen bond with the NH group of the Ile378 backbone (Fig. 3b). Moreover, the secondary amine of STM2457 (3b) establishes a salt bridge with Asp395 and a hydrogen bond with Ser511 (Yankova et al., 2021). STM2457 (3b) is a highly selective and potent METTL3 inhibitor exhibiting good pharmacokinetics and promising anticancer activity both *in vitro* and *in vivo*. In fact, STM2457 shows cancer-selective antiproliferative activity at micromolar levels in a panel of various AML cell lines (*e.g.*, EOL-1, HL60, Kasumi-1, MOLM-13, NOMO-1, OCI-AML2, OCI-AML3, and THP-1) along with increased lifespan, no significant weight variations, and no toxicity in AML PDX mouse models (Table S1) (Yankova et al., 2021).

Starting from STM2457 (3b), several METTL3 competitive inhibitors have been reported in patents filed by Accent Therapeutics, Inc (4a-h and 5a-d, Table S1) and Storm Therapeutics (6a-e, 7a-g, 8a-c and 9a-d, Table S1). All these compounds show antiproliferative activity in different cancer cell lines (*e.g*, Caov-3, Kasumi-1, and MOLM-13) at micromolar levels (Table S1). In addition, allosteric inhibitors (*e.g.*, CDIBA (10a) and its derivative 10h as well as eltrombopag (11) may represent a valid alternative to METTL3 SAM-competitive inhibitors impairing cell growth (Table S1) (Liao et al., 2022; Lee et al., 2022). Noteworthy, CDIBA (10a), its derivative 10h, and eltrombopag (11), have shown that METTL3-METTL14 possesses allosteric sites that can be exploited for modulation of the methyltransferase activity in future studies.

Recently, quercetin (12, Table S1) has been reported as a potential METTL3 inhibitor able to impair human pancreatic adenocarcinoma cell (*e.g.*, MIA PaCa-2 and Huh7) viability at micromolar concentration. Although quercetin (12) is able to fill the SAM adenosine binding pocket its action mode is still unknown (Table S1) (Du et al., 2022).

In summary, the introduction of these novel METTL3 inhibitors has been a breakthrough that moved epitransciptomics on the top-rank of new potential AML-therapeutic targets. Interestingly, further studies (Yankova et al., 2021) brought to the development of a STM2457 derived drug, named as STC-1. This METTL3 inhibitor entered phase-1 clinical trials in subjects with solid tumors on November 2022 in the USA, with primary estimation end of data collection in September 2023 (#NCT05584111).

10. Conclusions

The detection of large mutational profiles using NGS, and the dynamics of clonal evolution are the keys to reliably follow AML patients, monitor MRD levels and choose the best drugs in the physician armamentarium. NGS is enabling precision medicine at the molecular level; we anticipate that this will allow it to enter more and more trials and ultimately become a readily-available tool for physicians to make significant clinical decisions based on patients' individual characteristics. Besides the obtainment of CR after induction chemotherapy, it is becoming clear to physicians that the primary aim will be to maintain this CR state and obtain real disease eradication through the implementation of drugs tailored on each individual AML cancer profile.

Appropriate MRD monitoring will be instrumental to follow small disease traces and plan optimal therapeutic interventions. In order to fully achieve these goals, several impending challenges will have to be faced, such as increasing sensitivity and specificity, and obtaining full standardization and harmonization among centers, to find agreement on when and which biomarkers to test.

m^6A/METTL3 seems to represent a hub where disparate leukemia-related pathways converge, affecting the aggressiveness of AML cells and their sensitivity to therapy. In this sense, it could be reasonable to propose METTL3 as a novel prognostic marker for AML. Moreover, observing the comprehensive portrait of the aforementioned studies, the function of METTL3/14-m^6A in AML appears to be declined in the form of "one-to-many", since various different m^6A targets contribute at multiple levels in determining AML cell aggressiveness and therapy sensitivity. The unique common factor of these parallel routes is the control of m^6A methylation. Future studies evaluating MTase complex components expression in larger cohorts of AML patients could shed light on the prognostic potential of METTL3 expression in AML. Particularly, it would be interesting analyzing the expression levels of METTL3 in AML populations classified according to the new 2022 risk-classification of ELN, in order to highlight any possible correlation with a specific phenotype of disease. This could clarify if the evaluation of METTL3 expression levels in newly diagnosed AML would be helpful in AML risk stratification and consequently in best therapy regimen choice.

Risk categorization of AML is a continuously-refining model that gradually adsorbs the enormous variety of data coming from genetics, clinical trials of new developed drugs, and now gene expression profiles. With the unstoppable growth of information included in the decisional algorithm, the introduction of artificial intelligence as a support for clinicians has become nowadays more acceptable than the past. AML research has already adopted this new branch, producing several refining software/systems capable to analyze cases from clinical practice (Radakovich et al., 2020). This should not be considered as a substitution of the crucial role and responsibility of physicians, but as a boost speeding up their analytic capabilities, reaching more easily the most possible accurate diagnosis, prognosis, and therapy for AML. We anticipate that these aspects will be covered in the next few decades and will dramatically improve the clinical course of AML, along with all other onco-hematologic malignancies.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.amolm.2023.100023.

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