SEMMELWEIS EGYETEM

DOKTORI ISKOLA

# Ph.D. értekezések

## 3259.

# KRIZSÁN SZILVIA

**Onkológia**

című program

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# Next-generation sequencing-based genomic profiling of pediatric acute myeloid leukemia and inherited myeloid malignancies

**PhD thesis**


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Budapest
2025

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ALL | acute lymphoblastic leukemia |
| AML | acute myeloid leukemia |
| AMKL | acute megakaryoblastic leukemia |
| APL | acute promyelocytic leukemia |
| BCL2 | B-cell lymphoma 2 |
| BFM | Berlin-Frankfurt-Münster |
| BM | bone marrow |
| BMF | bone marrow failure |
| bZip | basic leucine zipper |
| CBF | core-binding factor |
| CD | cluster of differentiation |
| CIR | cumulative incidence of relapse f |
| CN | cytogenetically normal |
| CNS | central nervous system |
| COG | Children's Oncology Group |
| COSMIC | catalogue of somatic mutations in cancer |
| CR | complete remission |
| ddPCR | droplet digital polymerase chain reaction |
| DFS | disease-free survival |
| DNA | deoxyribonucleic acid |
| dbSNP | single nucleotide polymorphism database |
| DfN | different-from-normal |
| EFS | event-free survival |
| ELN | European Leukemia Network |
| EMA | European Medicines Agency |
| ESP | Exome Sequencing Project |
| FAB | French-American-British |
| FDA | Food and Drug Administration |
| FISH | fluorescence in situ hybridization |
| FLAG | fludarabine |
| FML | family |

| | |
|---|---|
| GnomAD | genome aggregation database |
| GO | gemtuzumab ozogamicin |
| HPC | hematopoietic progenitor cell |
| HR | high-risk |
| HSC | hematopoietic stem cell |
| HSCT | hematopoietic stem cell transplantation |
| ICC | International Consensus Classification |
| InDel | insertion-deletion |
| ITD | internal tandem duplication |
| LAIP | leukemia-associated immunophenotype |
| MDS | myelodysplastic neoplasm |
| MFC | multiparameter flow cytometry |
| MLL | mixed lineage leukemia |
| MR | medium-risk |
| MRD | measurable residual disease |
| NCCN | National Comprehensive Cancer Network |
| NGS | next-generation sequencing |
| PCR | polymerase chain reaction |
| r | rearrangement |
| OS | overall survival |
| pCT | post-cytotoxic therapy |
| RT-PCR | real-time polymerase chain reaction |
| RNA | ribonucleic acid |
| SBDS | Shwachman-Bodian-Diamond Syndrome |
| SNP | single nucleotide polymorphism |
| SNV | single nucleotide variant |
| SR | standard-risk |
| TARGET | Therapeutically Applicable Research to Generate Effective Treatments |
| TCP | thrombocytopenia |
| TKD | tyrosine kinase domain |
| VAF | variant allele frequency |
| WBC | white blood cell |

| | |
|---|---|
| WES | whole exome sequencing |
| WGS | whole genome sequencing |
| WHO | World Health Organization |

# I. INTRODUCTION

## I.1. Pediatric acute myeloid leukemia

Acute myeloid leukemia (AML) is a heterogeneous malignancy caused by the malignant transformation of the stem cell precursors of the myeloid lineage, characterized by the impaired maturation and uncontrolled proliferation of the leukemic blast cells. AML is a relatively rare disease; nevertheless, it accounts for the highest number of annual deaths from leukemias among adults, which demonstrates that it is one of the hardest to treat hematological malignancies (1). If AML is left untreated, it progresses rapidly and can have a fatal outcome within weeks. With the advent of molecular biology, we now have a better understanding of the biology of AML, which has ultimately led to more accurate diagnosis according to international standards, improved risk classification, and the development of novel therapies.

AML is a relatively rare malignancy, as it accounts for only 1.0% of all new cancer cases; however, in adults, it is the most common form of acute leukemia in the developed world, with an age-adjusted incidence of 4.1 per 100,000 people (2). AML is the disease of the elderly, as the median age at diagnosis is 68 years, with approximately 60% of patients diagnosed at ≥65 years of age; nonetheless, AML can be diagnosed at any age, from newborn to very elderly (2). Leukemia is the most common type of malignancy in childhood, accounting for 25 to 30% of cancers, of which acute lymphoblastic leukemia (ALL) is the most common subtype, as it constitutes 75% to 80% of cases. While pediatric AML is a rare disease as it is responsible for only 15% to 20% of cases, with an incidence of approximately seven cases per million children younger than 15 years per year, it accounts for a disproportionate amount of more than 40% of childhood leukemic mortality (1, 3-7). The frequency peaks in children under 4 years of age and then increases steadily until adulthood (8). The incidence of AML is similar in both sexes, with a slightly higher number in males (1). In Hungary, the annual incidence rate of AML among children is approximately ten, according to the Hungarian Childhood Cancer Registry (5).

Over the past decades, studies have shown a global rise in the incidence of AML, most likely due to the growing and aging of the population and also because of the increase in therapy-related leukemias as a consequence of higher incidence and improved survival

rates of cancer patients (9, 10). Over time, there has been a consistent rise in the incidence of pediatric AML, especially in the frequency of AML post-cytotoxic therapy (pCT) (1, 11, 12). AML is one of the most common secondary malignancies, as approximately 0.5-1.0% of children with malignancy develop myeloid neoplasms after cytotoxic chemotherapy or radiation therapy (13).

There has been a remarkable improvement in the survival rate of patients with AML due to intensified chemotherapeutic regimens, advancements in risk assessment based on cytogenetic and molecular genetic analysis, as well as more precise measurable residual disease monitoring (MRD), optimization of allogeneic hematopoietic stem cell transplantation (HSCT), and advances in supportive care (14). Nonetheless, AML patients still face a dismal prognosis, with a 5-year overall survival (OS) rate of 31.7% (2). However, compared to adults, children have a better prognosis as they have better tolerance of more intensive therapeutic regimens and more commonly have favorable risk genetic features. Fortunately, the OS rates of pediatric AML have markedly improved in the past decades. In the 1970s, 5-year OS was about 10%, while nowadays, it reaches around 70% in developed countries (Figure 1) (15-18). Still, compared to the treatment of ALL, where the 5-year OS of children now exceeds 90%, the survival rate of children with AML seems suboptimal (19, 20).

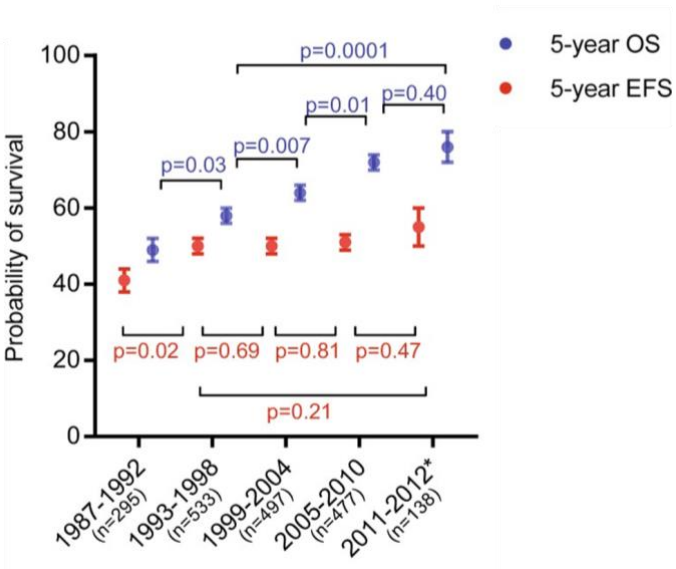



**Figure 1.** 5-year event-free survival (EFS) and OS of pediatric AML patients enrolled in the AML-BFM trials from 1987 to 2012 (15). 5-year OS increased steadily over the past decades; however, EFS improved less significantly.

The emergence of high-throughput sequencing technologies has led to the discovery of considerable molecular heterogeneity, observed across various patients and within individual cases, and deepened our understanding of the mechanisms driving leukemogenesis, clonal expansion, and resistance to treatment in leukemic cells. The wide application of next-generation sequencing (NGS) enabled detailed characterization of AML, leading to the identification of novel, recurrent molecular alterations, some of which serve as important prognostic and predictive biomarkers (21-23). The identification of targetable lesions has significantly expanded the therapeutic options available for treating AML in recent years. However, these advances are mainly seen in adult patients, and they are not fully reflected in the treatment of children with AML.

### I.1.1. Etiology of AML

Based on the etiology, AML traditionally can be categorized into de novo AML and secondary AML, with the latter including AML evolving from antecedent hematological disorder and AML post-cytotoxic therapy (AML-pCT) following exposure to chemotherapy or radiation therapy (24, 25). Most patients develop de novo AML without identifiable predisposing environmental exposures or inherited conditions. In a significant proportion of adult patients, AML can arise from an antecedent hematological disorder, including myelodysplastic neoplasm (MDS), myeloproliferative neoplasm, and aplastic anemia; though in children, it is rarely seen (26-28).

Among children with AML, the ratio of de novo AML is even higher than in adults, as about 95% of patients present without apparent etiology (7). Known risk factors for pediatric AML are exposure to benzene and ionizing radiation, as well as inherited conditions (29, 30). In the past decades, it has been increasingly recognized that AML cases can be associated with germline mutations, and recent studies have confirmed that germline predisposition to myeloid neoplasms is more common than previously appreciated (31, 32). Children more commonly have inherited susceptibility, predisposing them to the development of AML than adults (32).

The incidence of pediatric AML-pCT is rising due to the broader usage of platinum-based chemotherapy agents and the increasing number of long-term cancer survivors at risk (33-

35). Risk is exceptionally high among patients with prior exposure to alkylating agents (e.g. melphalan, cyclophosphamide), platinum compounds (cisplatin, carboplatin), topoisomerase II inhibitors (e.g. etoposide), and ionizing radiation (13, 33, 36). The majority of patients with AML-pCT have high-risk cytogenetic alterations and gene mutations. The prognosis for them is generally dismal, as 4-year OS rates were 25.5% and 37.8% for AML-pCT and de novo AML, respectively, according to the study of 2853 patients with newly diagnosed AML enrolled in multicenter trials of the German-Austrian AML Study Group(36-38). Notably, only a small proportion of patients exposed to cytotoxic therapy will eventually develop AML (33). Studies implicate that approximately 20% of AML-pCT may in fact be AML with germline predisposition caused by inherited mutations in the DNA repair genes associated with familial cancer predisposition syndromes (39, 40). Notably, the prevalence of pathogenic germline mutations is lower among adult patients with de novo AML, ranging from 7.2% to 13.6%, and in children, where it varies from 2% to 19.4% (31, 41-44). Another model suggests that patients with AML-pCT may have preexisting leukemic clones with *TP53* or *PPM1D* mutations prior to chemotherapy of primary malignancy, and these patients are at increased risk for the development of AML (45-48). Selective pressure from the cytotoxic therapy facilitates the emergence of these resistant clones, giving them an advantage over normal hematopoietic stem cells (47). However, in pediatric therapy-related myeloid neoplasm, the most frequently detected driver alterations were *KMT2A* rearrangements along with RAS/MAPK pathway mutations. *TP53* alterations were also common events, but unlike in adults, *TP53* mutations arose after chemotherapy (49).

### I.1.2. Molecular background of pediatric AML

The bone marrow is the site of hematopoiesis, where hematopoietic stem cells (HSC) and hematopoietic progenitor cells (HPC) undergo self-renewal and gradually differentiate into lineage-specific, unipotent progenitors that produce specific types of blood cells. Hematopoiesis under normal conditions is a well-regulated process shaped by the interplay of growth factors that facilitate cellular proliferation and transcription factors that activate certain genetic programs, leading to commitment to a specific lineage and terminal differentiation.

HSCs need to preserve a relatively constant number throughout life by carefully regulating the processes of self-renewal and differentiation (50). The deregulation of cellular functions may result in converting HSCs or HPCs into disease-causing stem cells, which may lead to hematopoietic malignancies, including leukemia. In AML, HSCs and HPCs acquire genetic alterations that confer pre-leukemic features, such as proliferative and survival advantage and impaired differentiation and apoptosis, early in the evolution of AML. At the time of diagnosis, the leukemic cells have already undergone a multistep evolutionary process originating from a prior premalignant clonal state (51).

In 2002, the "two-hit hypothesis" of leukemogenesis was proposed, which stated that AML is caused by at least two collaborating mutations belonging to different classes. Class I mutations (such as *FLT3*, *NRAS*, *KRAS*, *KIT*) confer proliferative and/or survival advantage to cells, while Class II mutations impair hematopoietic differentiation (e.g. *PML::RARA*, *RUNX1::RUNX1T1*, *KMT2A*-r, *CEBPA*) (52). The initiating mutations in AML are considered to be Class II alterations, while Class I mutations are mostly later events in leukemogenesis (52). Comprehensive genomic characterization of the AML genome revealed that the pathogenesis of AML is more complex than previously appreciated. Genes recurrently mutated in AML belong to various functional groups or pathways (21, 53). In a study conducted by The Cancer Genome Atlas Research Network, it was found that genetic alterations relevant to the pathogenesis of AML can be grouped into nine categories, including signaling (59%), DNA-methylation-related (44%), chromatin-modifying (30%), nucleophosmin (*NPM1*) (27%), myeloid transcription factor (22%), transcription factor fusions (18%), tumor suppressor (16%), spliceosome complex genes (14%) and cohesin complex (13%) (53).

Earlier studies revealed that AML is not a monoclonal disease but a collection of subclones with highly dynamic and diverse genetic identities (54). Two major types of mutation can be distinguished. Founding mutations are present in all leukemic cells, while subclonal mutations exist in only a fraction of cells. Mutations within the founding clone are stable, while mutations found in subclones can be gained or lost during progression (54). Single-cell mutation profiling has shown that during disease progression, treatment, or relapse, clonal evolution primarily occurs in linear and branching forms. Linear

evolution trajectory means the new clones arise from the stepwise acquisition of mutations, while in branching evolution, clones arise from one parental clone by acquiring new distinct mutations in each of the daughter clones, all of which can evolve in parallel (23, 55, 56).

#### *I.1.2.1. Karyotypic alterations in pediatric AML*

Chromosomal aberrations have a pivotal role in the diagnosis and risk stratification of AML as they are strong independent predictors of outcome. Recurrent cytogenetic alterations include balanced translocations, inversions, deletions, insertions, monosomies, and trisomies. The incidence of cytogenetic abnormalities is higher in children with AML compared to adult patients, as chromosome aberrations are detected in 75% to 80% of pediatric AML cases in contrast to 45% to 50% seen in adults with AML (4). Balanced chromosomal translocations are the most prevalent cytogenetic abnormalities found in pediatric AML, which result in the formation of chimeric fusion genes (57). Although a large variety of chromosomal alterations can be detected in AML, most pediatric AML cases can be assigned into distinct cytogenetic subgroups: 25% have t(8;21)(q22;q22)/*RUNX1::RUNX1T1*, inv(16)(p13;q22))/*CBFB::MYH11* or t(16;16)(p13;q22)/*CBFB::MYH11* (together referred to as core-binding factor (CBF)-AML); 12% have t(15;17)(q22;q21) /*PML::RARA*; 20% have rearrangements involving the *KMT2A* gene; 20% have no detectable chromosomal aberrations (normal karyotype AML), while the remaining 23% includes *NUP98* rearrangements, 12p13 abnormalities, and other rare balanced and unbalanced cytogenetic abnormalities(57). Cytogenetic alterations have significant age-associated variations; for example, the prevalence of *KMT2A* rearrangement is higher in younger patients, while CBF-AML is more common in adolescents (58). Several translocations, such as t(1;22)(p13;q13)/*RBM15::MRTFA*, t(7;12)(q36;p13)/*MNX1::ETV6* and t(11;12)(p15;p13)/*NUP98::KDM5A* occur almost exclusively in children and are rarely or never detected in adults (7, 57).

CBF-AML is the most frequent subtype of pediatric AML that can occur in all age groups, though it is uncommon under one year of age, and its incidence increases with age (57). The CBF complex is a heterodimeric transcription factor complex that includes a DNA-

binding alpha subunit from the Runt-related transcription factor (RUNX) gene family and a non-DNA-binding beta subunit, CBFb, which stabilizes the binding of the alpha subunit to DNA (59). Normally, RUNX1 and CBFB heterodimerize to DNA and recruit transcription factors that regulate the differentiation of hematopoietic cells. Both *RUNX1::RUNX1T1* and *CBFB::MYH11* fusion genes inhibit the normal function of the CBF complex in a dominant negative manner, leading to blockage of the myeloid differentiation. As both t(8;21) and inv(16) disrupt the CBF complex, they are grouped together in risk classification systems; however, several studies demonstrated considerable clinical and molecular heterogeneity within this group. Regarding morphology, t(8;21) is more commonly associated with 'AML with maturation,' while inv(16) is typically associated with 'myelomonocytic leukemia with aberrant eosinophils.' *RUNX1::RUNX1T1* and *CBFB::MYH11* rarely occur in isolation, as additional mutations or cytogenetic alterations are also detectable in most cases, suggesting that these gene fusions are insufficient to induce leukemia (59-61). Additional chromosomal aberrations are relatively common: loss of sex chromosome, del(9q), trisomy 8, abnormal(7q), and trisomy 4 occur the most frequently; however, the prognostic value of additional chromosomal alterations is still controversial (61-66). Regarding prognosis, *KIT* mutations did not affect the complete remission (CR) rate in children, but they affected OS, event-free survival (EFS), disease-free survival (DFS), and relapse rate, according to most studies. Nonetheless, the results are inconclusive, as three studies found that *KIT* mutations lack prognostic significance (67-69). The prognostic significance of *KIT* mutations may also differ between t(8;21) and inv(16) (70). Additionally, the site of mutation can also influence the prognostic impact, as *KIT* D816 mutation at exon 17 shows a strong adverse effect, while mutations at other sites have no noticeable effect on prognosis (71). An increased risk of relapse was observed in CBF-AML patients with *FLT3*-ITD mutations (66, 72). Contrary to that, *NRAS* mutations were independent predictors of favorable outcomes in children and adults in AML with *CBFB::MYH11* (61, 73). Both fusion genes are reliable markers for MRD monitoring and are associated with favorable outcomes, as the OS rate of patients exceeds 80%. Nevertheless, the incidence of relapse remains around 30% (7, 70).

Acute promyelocytic leukemia (APL) represents a unique disease entity formerly classified as AML-M3 based on the French-American-British (FAB) classification system. APL is characterized by the balanced translocation t(15;17)(q24.1;q21.2), leading to gene fusion between the promyelocytic leukemia (PML) gene and the retinoic acid receptor alpha (RARA). *PML::RARA* functions as a transcriptional repressor of both RARA and non-RARA target genes, leading to the blockage of differentiation and increased self-renewal of myeloid progenitor cells (74). APL accounts for 5% to 10% of pediatric AML patients, and its prevalence rises progressively with advancing age (58). Understanding the mechanism of pathogenesis and application of appropriate targeted therapy turned this particularly fatal disease into a highly curable one. The combination of all-trans-retinoic acid (ATRA) and arsenic trioxide (ATO) without conventional chemotherapy is the basis of modern treatment regimens of APL, while the use of traditional chemotherapy is restricted to only the induction phase for high-risk patients (75). Children with APL have the highest cure rates of pediatric AML, with OS near 95% and EFS of 90% (76, 77).

AML with *KMT2A* (formerly known as mixed lineage leukemia [MLL]) rearrangement is a heterogeneous AML subtype that occurs significantly more frequently in children, as 16% to 26% of patients showed *KMT2A* rearrangement (4, 78). Moreover, in the infant group, the rearrangement of *KMT2A* is the most common cytogenetic alteration, representing more than 50% of the cases (79). The abnormality is usually a reciprocal translocation between *KMT2A* and one of more than 120 other genes in distinct chromosomal loci (78, 80). *KMT2A* encodes a histone methyltransferase, which regulates gene transcription by methylation of histone H3 Lys4, inducing an open chromatin conformation. *KMT2A* rearrangements lead to oncogenic *KMT2A* fusion proteins, causing inappropriate histone modification and aberrant transcription activation, leading to upregulated expression of *HOXA/B* genes and *MEIS1* (81). The clinical outcome of patients with *KMT2A* rearrangement is highly variable and closely dependent on the fusion gene partner of *KMT2A* (78, 82, 83). The most extensive study on childhood AML with *KMT2A* rearrangements (*KMT2A*-r) was published recently, which shed light on the prognostic significance of different *KMT2A* rearrangements. This retrospective study confirmed the adverse outcome of the previously defined *KMT2A* fusions

(t(6;11)(q27;q23) *KMT2A::AFDN*, t(10;11)(p12;q23) *KMT2A::MLLT10*, t(10;11)(p11.2;q23) *KMT2A::ABI1*, t(4;11)(q21;q23) *KMT2A::AFF1* and t(11;19)(q23;p13.3) *KMT2A::MLLT1*), while describing three new recurrent *KMT2A*-r groups (i.e., Xq24/*KMT2A::SEPT6*, 1p32/*KMT2A::EPS15*, and 17q12), of which the former two were associated with better outcome (78, 84). The previously described favorable outcome of patients with translocation t(1;11)(q21;q23) *KMT2A::MLLT11* has not been confirmed in a recent comprehensive study (83). The most common *KMT2A* translocation was t(9;11)(p22;q23) *KMT2A::MLLT3* fusion occurring in 43% of patients. The clinical outcomes of patients with *KMT2A*::*MLLT3* are highly variable; therefore, risk stratification within this group can be refined further. FAB M5 morphology was associated with better outcomes, while trisomy 6 and MRD detected by flow cytometry at the end of induction 2 were independently associated with adverse prognosis (83).

Other rare but recurring chromosomal alterations in childhood AML include t(6;9)(p22;q34) *DEK::NUP214* that occurs in older children and is associated with a high risk of relapse, which may be improved by HSCT (4). The t(8;16)(p11;p13) *KAT6A::CREBBP* is present in only 1% of patients. Interestingly, it has an age-dependent impact on prognosis, as in very young children, this fusion gene is associated with spontaneous remission, advocating for a watch-and-wait strategy before initiating chemotherapy. On the other hand, older pediatric patients need intensive chemotherapy treatment (85). The t(16;21)(p11;q22) *FUS::ERG* is only present in about 0.5% of patients; however, it is noteworthy that it is associated with markedly inferior prognosis, with a 4-year EFS of 7% and a 4-year cumulative incidence of relapse (CIR) of 74%. Most relapses occurred within the first year after diagnosis, and interestingly, MRD status did not correlate with the risk of relapse, indicating that MRD is not a reliable predictor of relapse in this specific cytogenetic group. Patients with *FUS::ERG*-rearrangement require high-risk therapy, including HSCT in CR1, or even experimental therapy. (86). In children, the occurrence of unbalanced chromosomal alterations, such as monosomy of 7, monosomy 5/del5q, and aberrations of 12p is relatively low, affecting about 3% to 5% of cases; however, they are associated with adverse outcomes, similarly to adult cases (4).

The abovementioned chromosomal aberrations involve large chromosomal regions; therefore, conventional cytogenetics can detect them easily. Nevertheless, cryptic translocations (i.e. translocations undetectable by karyotyping) require more specialized techniques such as polymerase chain reaction (PCR) or fluorescence in situ hybridization studies (FISH), or nowadays optical genome mapping or RNA next-generation sequencing are also available options for detection. Several cryptic translocations have been discovered recently and shown to be prevalent in children with AML. For example, cryptic translocation of *NUP98* is among the most common recurrent translocations in pediatric AML, as it occurs in 4% of patients and is enriched in seemingly normal karyotype AML (87-89). Until now, more than 30 gene partners have been reported for *NUP98*, with *NUP98*::*NSD1* and *NUP98::KDM5A* being the most prevalent (89-91). *NUP98*::*NSD1*, encoded by the cryptic t(5;11)(q35;p15.5), is a frequent event in children with cytogenetically normal (CN) AML, as 16% of patients have this translocation compared with 2.3% seen in adults with CN AML (87). *NUP98*::*NSD1* is associated with a high frequency of *FLT3* and *WT1* mutations and dismal outcomes (87, 92). *NUP98::KDM5A* and *NUP98* translocations with other partners are also associated with treatment resistance and particularly adverse outcomes (91). *NUP98* plays a critical role in regulating gene transcription in the hematopoietic system. *NUP98* fusion protein interacts with chromatin-modifying complexes, including the MLL1 complex. The interaction between MLL and its cofactor menin has been shown to be critical in *KMT2A*-r and *NUP98*-r leukemia, and disruption of this association is under clinical evaluation as a potential treatment option. Recently published studies showed that menin inhibitors are therapeutically effective in *NUP98*-r AML (93, 94).

RNA sequencing of children with acute megakaryocytic leukemia (AMKL) has led to the identification of a cryptic inversion of chromosome 16, causing fusion between *CBFA2T3* and *GLIS2* genes in nearly 30% of children with AMKL without Down syndrome (90, 95). *CBFA2T3*::*GLIS2* fusion gene disrupts the balance of transcription factors essential for normal hematopoiesis, especially causing downregulation of *GATA1*, which is vital for megakaryocytic differentiation. Subsequent studies have found that *CBFA2T3*::*GLIS2* fusion is not limited to AMKL, as it can be detected in AML with seemingly normal karyotype and other karyotypic alterations (96, 97). The prevalence of *CBFA2T3*::*GLIS2*

is about 2% in pediatric AML, and it is highly enriched in younger patients, as nearly 12% of infants under the age of 1 harbor this fusion (97). *CBFA2T3*::*GLIS2* is associated with resistance to conventional therapy and a high cumulative incidence of relapse even after HSCT (96-98). The absence of recurrent cooperating mutations in this highly aggressive disease indicated that this cryptic fusion alone could lead to malignant transformation (97). Given the dismal outcome with standard therapies, targeted therapies are being explored. Examination of the transcriptome of AML patients uncovered that *FOLR1* encoding folate receptor alpha is overexpressed in *CBFA2T3*::*GLIS2* AML, and it can be targeted by luveltamab tazevibulin. Luveltamab tazevibulin is a FOLR1-antibody-drug conjugate demonstrated to have potent anti-leukemia activity in *CBFA2T3*::*GLIS2* cell lines (99).

#### *I.1.2.2. Somatic mutations in pediatric AML*

Cytogenetic alterations play a dominant role in the pathogenesis of pediatric AML; however, disease-associated recurrent mutations are also detected frequently in patients, especially in those with normal karyotype. The *FLT3* gene is commonly mutated in pediatric AML, though it is rarely seen in children under two years of age. However, its prevalence increases with age, occurring in 20 to 25% of adolescents (100, 101). *FLT3* encodes a receptor tyrosine kinase normally expressed by immature hematopoietic stem and progenitor cells. It significantly influences the initial phase of myeloid and lymphoid lineage development by regulating their growth, maturation, and cell survival (102). *FLT3* mutation causes ligand-independent constitutive activation of the receptor and drives downstream signaling pathways. Traditionally, *FLT3* mutations are classified into two main types: internal tandem duplications (ITD) in the juxtamembrane domain and missense mutations in the tyrosine kinase domain (TKD) (most commonly at codons 835 and 836), both resulting in aberrant *FLT3* activity (102). Several non-ITD/TKD mutations with aberrant *FLT3* activation have recently been identified in children (103). *FLT3*-ITD mutations are associated with poor prognosis, especially in patients with a high allelic ratio (>0.5). *FLT3*-ITD mutations have very adverse outcomes when treated with chemotherapy alone. Nonetheless, recently, the allelic ratio has been eliminated from the risk stratification of adults with AML because of the difficulty of standardization (104). A newly published study by Tarlock et al. revealed that the clinical outcomes of pediatric

patients with *FLT3*-ITD mutations are determined by co-occurring mutations rather than the allelic ratio of *FLT3*-ITD mutations (105). *FLT3*-ITD mutated patients with favorable-risk alterations such as t(8;21), inv(16), *NPM1*, or *CEBPA* mutations had significantly superior outcomes compared to patients with high-risk genetics, including mutations of *WT1*, *UBTF*, or *NUP98*::*NSD1* (105). The prognostic effect of *FLT3*-TKD mutations is inconsistent (106). *FLT3* mutations are unstable during disease progression as they can be gained or lost at relapse or progression, which suggests that *FLT3* mutations are secondary events in leukemogenesis (23, 107).

*NPM1* mutations are common in AML, as the prevalence is 30% in adult AML patients; however, it is relatively rare in pediatric AML, especially in younger children (21, 100). *NPM1* mutations are notably higher in AML with normal karyotype, affecting 40% to 50% of adults and about 10% in pediatric cases (108, 109). *NPM1* encodes a histone chaperone protein mainly localized in the nucleolus, which, under normal circumstances, shuttles between the nucleus and cytoplasm. *NPM1* has several functions: cell cycle regulation, ribosome biogenesis, and DNA damage response. The disruption of *NPM1* results in the cytoplasmic dislocation of *NPM1*, leading to the activation of oncogenic *HOXA* and *HOXB* cluster genes and ultimately resulting in leukemogenesis (110). The most frequent mutation of *NPM1* is an insertion of 4 base pairs in exon 12, leading to a frameshift in the region encoding the C-terminal of the *NPM1* protein. *NPM1* mutations are associated with better outcomes, resulting in reduced risk of relapse and higher overall survival rate (4, 104, 109). Menin inhibitors seem to be promising agents in the therapy of *NPM1*-mutated AML as they target the HOX/MEIS transcriptional program critical in the leukemogenesis of *NPM1*-mutated AML (111).

Mutation of *CEBPA* occurs in about 5% of patients and is enriched in patients with normal karyotypes (100). *CEBPA* encodes a transcription factor that regulates myelopoiesis and granulocyte differentiation (112). Two distinct mutational patterns often occur together on different alleles: the first one is typically a frameshift mutation that clusters at the N-terminal and involves the transactivation domains; the second one is mostly an in-frame mutation at the C-terminal affecting the basic leucine zipper (bZip) region. Germline mutations predominantly cluster within the N-terminal (112). Biallelic *CEBPA* mutations

have been associated with favorable outcomes; nevertheless, recently published studies showed that patients with *CEBPA*-bZip mutations show favorable outcomes irrespective of monoallelic or biallelic mutational status (112-114).

Large-scale next-generation sequencing studies have significantly advanced our understanding of the complex genomic landscape of AML. From these studies, it became evident that the genetic landscape of pediatric and adult AML is vastly different, especially concerning the incidence and type of mutations. Epigenetic modulators such as *DNMT3A*, *IDH1*, *IDH2*, *ASXL1*, *TET2*, and *EZH2* are frequently altered in adults, though they are extremely rare in children (21, 53, 100, 101). Similar observations were made with mutations of genes involved in RNA splicing (*SF3B1*, *SRSF2*, *U2AF1*) as their prevalence was almost 30% in adults, while they were rarely detected in the pediatric cohort (21, 100, 115). Mutations enriched in the pediatric patients were *KRAS*, *NRAS*, *PTPN11*, and *WT1* (100, 101). The number of somatic mutations in children with AML was significantly lower than in adult AML (5 per pediatric sample versus 10-13 per adult sample) (100).

Despite the comprehensive characterization of the genome of pediatric AML in the past decades, novel recurrent mutations can be discovered even nowadays. In 2021, Stratmann et al. identified in-frame internal tandem duplications in the *UBTF* gene as a recurrent event in children with relapsed AML, as it was found in 12% (3/25) of pediatric cases (116). Tandem duplication (TD) in *UBTF* may be a previously underappreciated lesion in pediatric AML, as most gene panels do not include this specific gene, and the length of these duplications poses a challenge for most current NGS variant callers to detect (116). In 2022, Umeda et al. performed a more comprehensive genetic and transcription profiling of relapsed pediatric AML cases where they detected *UBTF*-TD in 8.8% of patients, making it the third most common molecular feature in relapsed pediatric AML cohort; that is striking, considering it has rarely been reported in the literature before (117-119). It was shown that patients with *UBTF*-TD remained MRD-positive at the end of the first induction treatment. Intermediate-risk cytogenetic abnormalities (i.e., normal karyotype or trisomy 8) with the co-occurrence of *FLT3*-ITD or *WT1* mutations are often observed in *UBTF*-TD AML cases. The co-occurrence of *FLT3*-ITD with *UBTF*-TD was

associated with unfavorable clinical outcomes. *UBTF*-TD was stable as the disease progressed and could be detected in the founding clone (117). Extended analysis of de novo pediatric AML cases identified *UBTF*-TD in 4.3% (45/1,053) of de novo pediatric patients, and all these cases lacked a concurring recurrent fusion oncoprotein (117). Given the clonal nature of *UBTF*-TDs and their lack of co-occurrence with other oncogenic drivers, it is plausible that *UBTF*-TDs could represent a new subtype-defining lesion in AML (120).

### I.1.3. Diagnosis and classification of AML

AML has a heterogeneous clinical presentation with nonspecific symptoms that make recognition challenging. Signs and symptoms of AML result from leukemic infiltration of the bone marrow and extramedullary sites. Patients commonly present with fatigue, loss of appetite, recurrent fever, bone pain, swollen lymph nodes, or physical signs of anemia (fatigue, pallor, headache, dyspnea on exertion), neutropenia (infections or fever), or thrombocytopenia (easy bruising, petechiae). The most common symptoms are caused by the excessive proliferation of leukemic blasts in the bone marrow, preventing the normal production of red blood cells, platelets, and neutrophils. Disseminated intravascular coagulation can occur in all subtypes of AML; however, it is much more frequent in APL (104). Leukemic blasts can infiltrate extramedullary sites, leading to lymphadenopathy, hepatomegaly, splenomegaly, disease in the skin (leukemia cutis), gingiva, orbit, and rarely, testicular involvement can be observed (104). Patients with high white blood cell counts might exhibit symptoms indicative of leukostasis, most often affecting the lungs and brain.

Diagnosis of AML is suggested by the complete blood count with differential showing pancytopenia, while examination of the bone marrow aspirate or biopsy can establish the diagnosis. The diagnosis and classification of AML are based on morphological, cytochemical analyses, immunophenotyping by flow cytometry, conventional cytogenetic analysis, FISH, and molecular genetic testing. Immunophenotyping is a rapid method to determine lineage and distinguish AML and ALL. Multiparameter flow cytometry (MFC) identifies lineage and degree of maturation of leukemic blasts based on

cell surface and intracellular markers. The minimum antibody panel requirement to fulfill WHO criteria for diagnosis of AML includes CD34, CD117, CD11b, CD11c, CD13, CD14, CD15, CD33, CD64, CD65, intracellular myeloperoxidase, I-lysozyme, CD41, and CD61 (121). MFC plays a crucial role in diagnosis and the assessment of response to treatment via the detection of MRD. Leukemic cells can present with an aberrant antigen expression pattern called leukemia-associated immunophenotype (LAIP) that can be detected at diagnosis and tracked during follow-up. The primary benefit of MRD assessment through flow cytometry is its applicability to more than 90% of patients with AML (122).

Conventional cytogenetic analysis is essential in evaluating AML, as identifying AML-defining genetic alterations is crucial for the appropriate classification. If karyotyping fails, FISH or real-time (RT) PCR are alternative methods to detect specific gene rearrangements and chromosomal abnormalities. Identification of structural or numerical chromosomal aberrations is essential as they can be closely related to prognosis and survival, or they can lead to special treatment, as in the case of APL with t(15;17)(q24;q21) *PML::RARA*. In young children, it is essential to search for specific translocations that appear in pediatric AML in a significantly higher frequency such as t(7;12)(q36;p13) *MNX1::ETV6*, t(11;12)(p15;p13) *NUP98::KDM5A* and t(1;22)(p13;q13) *RBM15::MRTFA* (7).

Molecular genetic testing must detect all the genetic abnormalities necessary to establish diagnosis, risk stratification, and identify actionable therapeutic targets. Molecular screening needs to cover both gene mutations and gene rearrangements. Patients with *NPM1* mutation and CBF-AML are recommended to undergo a baseline molecular assessment by RT-PCR or droplet digital PCR (ddPCR) to facilitate MRD monitoring following treatment (4, 107). In cases where AML with germline predisposition is assumed, comprehensive testing of predisposing genes should be performed (4, 42, 123). All pediatric patients must undergo a lumbar puncture with cerebrospinal fluid sampling to rule out central nervous system (CNS) disease, contrary to the practice seen in adults, where only patients with CNS symptoms are tested (4).

Historically, AML has been classified based on morphology and defined according to the French-American-British (FAB) classification system established in 1976. The FAB classification utilizes criteria related to morphology and cytochemistry that is influenced by the cell lineage and differentiation status. Nevertheless, due to the progress in understanding the genetic background of acute leukemia, the latest classifications place more emphasis on molecular criteria. Recently, novel AML classifications (Fifth edition of the World Health Organization [WHO] Classification of Haematolymphoid Tumours and International Consensus Classification [ICC] of Myeloid Neoplasms and Acute Leukemias) have been proposed (Table 1).

**Table 1.** Classification of AML according to the 5th Edition of WHO Classification of Haematolymphoid Tumours

| **Acute myeloid leukaemia with defining genetic abnormalities** |
|---|
| Acute promyelocytic leukaemia with *PML::RARA* fusion |
| Acute myeloid leukaemia with *RUNX1::RUNX1T1* fusion |
| Acute myeloid leukaemia with *CBFB::MYH11* fusion |
| Acute myeloid leukaemia with *DEK::NUP214* fusion |
| Acute myeloid leukaemia with *RBM15::MRTFA* fusion |
| Acute myeloid leukaemia with *BCR::ABL1* fusion |
| Acute myeloid leukaemia with *KMT2A* rearrangement |
| Acute myeloid leukaemia with *MECOM* rearrangement |
| Acute myeloid leukaemia with *NUP98* rearrangement |
| Acute myeloid leukaemia with *NPM1* mutation |
| Acute myeloid leukaemia with *CEBPA* mutation |
| Acute myeloid leukaemia, myelodysplasia-related |
| Acute myeloid leukaemia with other defined genetic alterations |
| **Acute myeloid leukaemia, defined by differentiation** |
| Acute myeloid leukaemia with minimal differentiation |
| Acute myeloid leukaemia without maturation |
| Acute myeloid leukaemia with maturation |
| Acute basophilic leukaemia |
| Acute myelomonocytic leukaemia |
| Acute monocytic leukaemia |
| Acute erythroid leukaemia |
| Acute megakaryoblastic leukaemia |

In the newest edition of the WHO classification, the most profound changes occurred in AML, myelodysplasia-related (formerly known as AML with myelodysplasia-related changes). Namely, the criterion of morphological dysplasia has been omitted due to its lack of prognostic relevance, and a new mutation-based molecular definition has been introduced, including a set of 8 genes (*ASXL1*, *BCOR*, *EZH2*, *SF3B1*, *SRSF2*, *STAG2*, *U2AF1*, *ZRSR2*). Therefore, the presence of one or more cytogenetic or molecular abnormalities and/or history of MDS or MDS/MPN is required for diagnosing AML, myelodysplasia-related, without the need for morphological dysplasia (121).

### I.1.4. Prognostic factors and risk stratification in pediatric AML

Accurate prognostication is one of the key factors for the successful treatment of AML. Therefore, the intensity of post-remission therapy is mainly assigned according to the risk of relapse to avoid excess toxicities. The outcome of AML varies widely, and both patient-related and disease-related factors influence an individual's prognosis. Patient-related pretreatment prognostic factors include age, performance status, and the presence of clinically significant comorbidities. Age at diagnosis is prognostic as the survival rate of children is superior compared to adolescents and young adults, while the clinical outcome of older adults with AML is particularly dismal. Older individuals with AML have a lower chance of achieving CR and face shorter disease-free survival (104). Retrospective analysis of children (2 to <13 years), adolescents (13 to <21 years), and young adults demonstrated significantly inferior 5-year EFS with increased age at diagnosis (124).

Disease-related factors include the leukemic cells' cytogenetic and molecular genetic profile and the quality of response to treatment. Establishing the molecular characteristics of AML is crucial as both cytogenetics and molecular genetics are significant independent predictors of prognosis and are the mainstay of the WHO classification of AML. Risk classification of pediatric AML varied slightly between study groups, as there were no published recommendations specific to children with AML. In 2012, an evidence-based consensus recommendation for diagnosing and managing AML was developed (4). Risk stratification relies heavily on recurrent cytogenetic alterations, with a handful of

molecular genetic alterations. Genetic abnormalities with favorable risk includes t(8;21)(q22;q22.1) *RUNX1::RUNX1T1*, inv(16)(p13.1q22) or t(16;16)(p13.1;q22) *CBFB::MYH11*, t(15;17)(q22;q21) *PML::RARA*, normal karyotype with *NPM1* or biallelic *CEBPA* mutation, t(1;11)(q21;q23) *KMT2A::MLLT11* and *GATA1* mutation, while complex karyotype, monosomy 7, monosomy 5/5q-, inv(3)(q21q26.2) or t(3;3)(q21;q26.2) *RPN1::MECOM*, t(6;9)(p23;q34) DEK::NUP214, t(7;12)(q36;p13) *MNX1::ETV6*, t(4;11)(q21;q23) *KMT2A::AFF1*, t(5;11)(q35;p15.5) *NUP98*::NSD1, t(6;11)(q27;q23) *KMT2A::AFDN*, t(10;11)(p12;q23) *KMT2A::ABI1*, t(9;22)(q34;q11.2) and *WT1* mutation with *FLT3*-ITD are associated with poor outcomes (4). Approximately two-thirds of patients are categorized as intermediate risk, which comprises patients with normal karyotypes, chromosome 11q23 abnormalities, AMKL, and others with varying clinical outcomes (58). This heterogeneity implies the presence of additional genetic alterations not identified by cytogenetic analysis but still relevant in the clinical outcome of patients with AML.

Risk classification systems of adult AML patients continuously incorporate novel findings of comprehensive genomic profiling, such as those defined by the newest European Leukemia Network (ELN) guideline (104). Unfortunately, adult risk classifications cannot be entirely transferred to pediatric cases, as the cytogenetic and genomic landscapes of pediatric and adult AML are fundamentally different (100, 115). Several novel driver alterations, specific for pediatric AML, have recently been identified. Nevertheless, these novel findings have not yet been included in the latest risk stratifications. However, in 2024, Umeda et al. published a novel comprehensive prognosis-related molecular classification system of pediatric AML with 23 molecular categories (125). By applying transcriptome and genome profiling, they could classify and risk-stratify 91.4% of patients instead of the 68.5% covered by the WHO classification. This extensive framework for molecular diagnostics and prognostic systems could be the foundation for risk assessment and refinement of treatment strategies of pAML in the future.

Assessment of response to treatment is widely used to risk stratify patients as it is a strong determinant of future outcomes. Complete remission (CR) is traditionally defined as <5%

blasts in the bone marrow. More than 90% of children with AML achieve CR after induction therapy; nevertheless, it is important to note that 30 to 40% of patients will eventually experience overt relapse due to residual undetected disease. Therefore, the need for more accurate methods emerged to evaluate treatment response, which led to the determination of MRD. Traditionally, two MRD detection methods have been used in routine clinical practice: MFC and PCR-based approach; however, next-generation sequencing has recently been increasingly used in research studies. Both MFC-based and real-time (RT) PCR-based MRD measurements are highly prognostic for long-term survival. Two MFC-MRD methods exist to target leukemia cells: the LAIP method, which is used by several study groups, and the different-from-normal (DfN) approach utilized in the Children's Oncology Group (COG) studies. With the LAIP approach a patient-specific antigen combination is selected at diagnosis, while a standardized panel is employed in the DfN approach, which has the potential to be applicable to every patient irrespective of the immunophenotype of leukemic blast at diagnosis and has the advantage of independence from the stability of a diagnostic LAIP, enabling the detection of blasts even if an immunophenotypic shift occurs. Regardless of the method applied, MRD positivity at the end of induction therapies is considered the most reliable predictor of poor outcomes (14). The PCR-based method is used to monitor specific translocations and mutations quantitatively. Validated molecular MRD targets for quantitative RT-PCR are *PML::RARA*, *RUNX1::RUNX1T1*, *CBFB::MYH11*, and *NPM1* mutation (107). The main advantage of quantitative RT-PCR is its increased sensitivity compared to MFC. The most significant issue of molecular MRD is that its application is limited in children, as MRD targets are present in a minority of pediatric AML patients (7, 126, 127). Consequently, due to its broad applicability, MFC-MRD is the preferred method for MRD monitoring in pediatric AML, notwithstanding the fact that its sensitivity is lower (0.1-0.01%) than molecular MRD (14).

### I.1.5. Treatment of AML

Treatment of pediatric AML differs in many ways between the international cooperative groups. There is no consensus on the cumulative doses of drugs, the kind of anthracycline used, the number and intensity of treatment blocks, the intrathecal chemotherapy used for CNS prophylaxis, and the indication for HSCT. Nevertheless, regardless of the varying

strategies, the results are reasonably comparable. The prognosis of children with AML has dramatically improved during the past decades. CR rates as high as 80% to 90%, EFS rates up to 50%, and OS rates of 60% to 70% are now reported (4). The therapeutic armamentarium of pediatric AML has remained essentially unchanged. Still, advancements in treatment delivery and improvement in supportive care have facilitated the use of optimally intensive therapy with reduced risk of morbidity and mortality. Better salvage therapy following relapses has also played an essential role in improving OS. Nevertheless, traditional approaches are likely to have reached their limits, as reflected in the modest improvement in event-free survival (EFS) (15). Therefore, refinements in risk stratification and integrating new molecular-targeted therapies into current treatment protocols are essential.

The use of multi-agent combination chemotherapy continues to be the cornerstone of treatment for AML. Current management includes induction chemotherapy with the aim of reaching remission, followed by either consolidation chemotherapy or HSCT to prevent future relapse (4). Therapy for pediatric AML has been extrapolated from adult AML regimens. Since 1973, the standard of care induction therapy for adult AML patients has been the combination of cytarabine and anthracycline. With the "7+3 regimen" cytarabine is given by continuous intravenous infusion for 7 days, while an anthracycline is administered for 3 days (128). Like the treatment of adult patients with AML, standard induction chemotherapy in children is also based on the "7+3" regimen, but often a third drug (e.g. etoposide) is added. Anthracyclines are the key elements in the treatment of AML, but their total cumulative dose needs to be limited due to their cardiotoxic side effects (129). CNS involvement in adult AML patients is relatively rare; however, in children with newly diagnosed AML, the presence of AML cells in the cerebrospinal fluid has been reported to be around 30% (130, 131). At relapse, 10% of patients had CNS involvement, and without CNS prophylaxis, CNS involvement at relapse was 20% (132-134). Due to the high frequency of CNS involvement in children with AML and the challenging nature of management of patients with CNS disease, intrathecal prophylaxis is indicated in children (4).

Several studies investigated how the outcome of children with AML could be further improved; however, most trials have failed to show a positive impact on survival. Administration of high-dose cytarabine, different anthracyclines, or alternative cytotoxic agents yielded no positive results. An exception is the incorporation of gemtuzumab ozogamicin (GO), an anti-CD33 antibody-drug conjugate. CD33 is highly expressed on leukemic cells in more than 80% of patients with AML (14). Consequently, GO can be used for a broad range of patients, which makes it an appealing option. In the COG AAML0531 randomized study, the addition of GO to induction therapy significantly improved the EFS of pediatric patients with newly diagnosed AML (135). GO is approved by the FDA for the treatment of de novo AML in patients aged one month or older, in combination with standard chemotherapy, as well as patients with relapsed AML, and is considered a standard treatment in the United States (136). Contrary to that, GO is only approved in Europe for treating patients with newly diagnosed AML aged 15 years and older (137).

Consolidation therapy is provided to all patients who achieve morphological CR to consolidate the remission status. Drugs that are being used are almost the same as induction chemotherapies, consisting mainly of cytarabine with or without anthracyclines and/or other additional drugs (4, 15). Many of the questions regarding post-induction chemotherapies still need to be solved, including the number of courses and the addition of other cytotoxic drugs.

Maintenance therapy is established to be beneficial in many cancers (including ALL); however, studies in pediatric AML did not confirm a survival benefit with maintenance therapy (138). Thus, it is not part of the standard of care in most treatment protocols, except for the Berlin-Frankfurt-Münster (BFM) AML group. The BFM-AML group has applied maintenance chemotherapy for decades; nonetheless, the overall results do not differ significantly from those of other groups that do not use maintenance (134). In the AML-BFM 2012 study, patients were allocated randomly to receive maintenance to evaluate whether specific molecular subsets could benefit. The COG clinical trial AAML1031 has been evaluating the potential benefit of maintenance with sorafenib, an *FLT3* inhibitor, to find that sorafenib improved the event-free survival and relapse rate of

children with AML (139). Maintenance therapy is routinely used in selected patient cohorts in the adult setting. Maintenance therapy with oral azacitidine (CC-486) and hypomethylating agents (azacitidine, decitabine) is generally recommended for patients with non-CBF-AML who are not eligible for allogeneic HSCT based on the positive results of randomized trials (140, 141). *FLT3* inhibitors (sorafenib, midostaurin, gilteritinib, quizartinib) can also be offered for maintenance after allogeneic HSCT in remission for patients with a history of *FLT3*-ITD mutation (142).

Allogeneic HSCT constitutes the most potent anti-leukemic therapy in AML. Donor-derived cytotoxic immune cells eliminate residual leukemia cells after a prior conditioning regimen known as the graft-versus-leukemia effect. Indication for allogeneic HSCT during first remission depends on the risk-benefit ratio based on the disease's cytogenetic and molecular genetic features and response to initial therapy. Treatment-related mortality is approximately 10% to 15% if the patient is transplanted in first complete remission (CR1) or second complete remission (CR2). Allogeneic HSCT should be taken into account if the probability of relapse without the procedure is predicted to be >35% (143). Therefore, HSCT is only recommended in CR1 for patients with unfavorable genetic alterations or inadequate response to induction therapy. For relapsed or refractory AML patients, allogeneic HCT offers the best chance for cure (144). Due to better prevention and treatment of infections and graft-versus-host disease, outcomes following transplant continue to improve, leaving relapse as the main reason for treatment failure post-HSCT (145).

#### *I.1.5.1. Targeted therapy in pediatric AML*

Enhanced insights into the pathogenesis of AML, along with progress in molecular genomic technologies, are facilitating the identification of novel drug targets and the development of tailored, risk-adapted treatment approaches. Since 2017, the FDA has approved 12 new drugs for the treatment of adult patients with newly diagnosed and relapsed/refractory AML, including: FLT3 inhibitor midostaurin, gilteritinib, and quizartinib; IDH inhibitor enasidenib, ivosidenib, and olutasidenib; BCL2 inhibitor venetoclax; hedgehog inhibitor glasdegib; DNMT inhibitor CC486; menin inhibitor

revumenib; CD33-directed antibody-drug conjugate gemtuzumab ozogamycin; and liposomal daunorubicin and cytarabine combination CPX-351. Unfortunately, therapies for AML are predominantly developed for adult patients; therefore, the utility and availability of novel targeted therapies are highly limited for children. Several genetic alterations have been identified that have the potential to be targeted by novel agents, and many of these targets are relevant for both adults and children.

The anti-CD33 antibody-drug conjugate GO has been the only available approved targeted agent in the treatment of pediatric AML until the very recent approval of menin inhibitor revumenib. Phase 3 COG AAML0531 study showed that adding GO to induction significantly improved the EFS of children with newly diagnosed AML (135, 146). GO is currently approved by the FDA for the treatment of newly diagnosed AML patients aged ≥1 month, in combination with standard chemotherapy, as well as of patients with relapsed AML, and is regarded as a standard of care in the United States. Contrarily, GO is only approved in Europe for treating newly diagnosed patients with AML aged ≥15 years (137). Other agents targeting CD33 are also under investigation in clinical trials, such as CD33-targeted chimeric antigen receptor T cells and CD33xCD3 bispecific antibodies.

Mutations of *FLT3* - encoding a receptor tyrosine kinase - are one of the most common somatic mutations in AML; thus, it is a promising target for therapeutic intervention. *FLT3* inhibitors can be categorized into two generations according to their specificity for *FLT3* and into types 1 and 2 based on their mechanism of action. First-generation *FLT3* inhibitors (i.e., midostaurin and sorafenib) target a broader spectrum of tyrosine kinases, leading to more off-target effects. In contrast, second-generation *FLT3* inhibitors (i.e. quizartinib, crenolanib, and gilteritinib) have enhanced selectivity for *FLT3*, resulting in fewer off-target effects. Type 1 *FLT3* inhibitors (midostaurin, gilteritinib, crenolanib) are active against both *FLT3*-ITD and *FLT3*-TKD mutations. Type 2 inhibitors (quizartinib, sorafenib) are effective only against *FLT3*-ITD mutations (147). COG trial AAML1031 evaluating sorafenib in combination with chemotherapy in pediatric AML with *FLT3*-ITD mutations showed favorable effects on clinical outcomes (139). Phase 3 trial testing

gilteritinib in combination with chemotherapy in de novo AML with activating *FLT3* mutations and studies with quizartinib for relapsed/refractory AML are ongoing (147).

B-cell lymphoma 2 (BCL2) is an anti-apoptotic protein linked to the pathogenesis of hematological malignancies. Venetoclax is a selective BCL2 inhibitor that induces the apoptosis of leukemic cells. In adult patients with AML, venetoclax combined with azacitidine and hypomethylating agents has shown significant efficacy (148). Several ongoing phase 1/2 trials evaluate venetoclax in combination with high-dose cytarabine, hypomethylating agents, and CPX-351 for children with R/R AML (147).

Promising new agents in the treatment of pediatric AML are menin inhibitors. Ongoing phase 1/2 studies, including patients with *KMT2A*-rearranged and *NPM1*-mutated acute leukemia, have shown encouraging clinical responses with the menin inhibitors SNDX-5613 (revumenib) and KO-539 (ziftomenib), which target the *KMT2A* fusion protein complex (149, 150). In November 2024, revumenib was approved by the FDA for relapsed/refractory acute leukemia with *KMT2A* translocation in adult and pediatric patients 1 year and older.

### I.2. Hereditary myeloid malignancies

Familial predisposition to hematopoietic malignancies was initially described as a rare entity; however, several novel predisposition genes have been identified due to the increased use of NGS. Recently published studies using high-throughput sequencing revealed that germline predisposition to myeloid neoplasms is more common than previously appreciated, as an estimated 5% to 10% of patients with hematological malignancy have an underlying genetic predisposition (31, 32, 151-153). This rate can be even higher in specific patient subsets; for example, among adolescent and young adults with MDS with chromosome 7 abnormalities related to germline *GATA2* and SAMD9/SAMD9L syndromes, the frequency of hereditary hematopoietic malignancies can be as high as 50% (153). All patients with hematopoietic malignancy should be assessed for underlying germline predisposition. However, in clinical practice, patients are offered genetic counseling and testing if germline predisposition is highly suspected.

The following few aspects can raise suspicion of germline disorder: (1) long-standing history of cytopenia, (2) history of previous malignancy, (3) diagnosed with malignancy at an unusually young age, (4) frequent or atypical infections, (5) physical features indicative of germline predisposition syndrome (abnormal nails or hair) (6) family history of bleeding, easy bruising, cytopenias or hematopoietic / young-onset solid tumors (123). Myeloid neoplasm with germline predisposition has been included in the WHO classification as a unique entity with three main subtypes: myeloid neoplasm with germline predisposition (1) without a pre-existing platelet disorder or organ dysfunction (*CEBPA*, *DDX41*, *TP53* variants), (2) with pre-existing platelet disorder (*RUNX1*, *ANKRD26*, *ETV6* variants) and (3) with potential organ dysfunction (121, 154). The latter category was extended in the 5$^{th}$ edition of the WHO classification, including germline GATA2 variant, inherited bone marrow failure syndromes, telomere biology disorders, RASopathies, MIRAGE syndrome, SAMD9L-related Ataxia Pancytopenia Syndrome, and Bloom syndrome (Table 2) (121).

The diagnosis of hereditary predisposition to myeloid neoplasms has profound clinical significance for the patients and their relatives, as it influences therapeutic decision-making such as donor selection for allogeneic HSCT, the choice of conditioning regimen before transplantation, appropriate genetic counseling, and cancer surveillance for relatives at-risk (155).

Inherited myeloid neoplasms show significant heterogeneity in penetrance, age of onset, and clinical characteristics (156). People carrying variants linked to germline predisposition have an elevated lifetime risk of developing myeloid malignancies. The clinical outcome of germline variant carriers can differ widely even within family members with the same variant, suggesting that acquiring somatic mutations influences individuals' risk for developing hematopoietic malignancy (157). Despite advances in the identification of germline variants, our understanding of the pathogenesis – including the additional somatic mutational landscape presumably responsible for variability in penetrance - and recognition of individuals at the highest risk for developing myeloid malignancies remains incomplete.

**Table 2.** WHO classification of myeloid neoplasms associated with germline predisposition.

| **Myeloid neoplasms with germline predisposition without a pre-existing platelet disorder or organ dysfunction** |
|---|
| • Germline *CEBPA* P/LP variant (*CEBPA*-associated familial AML)<br>• Germline *DDX41* P/LP variant<br>• Germline *TP53* P/LP variant (Li-Fraumeni syndrome) |
| **Myeloid neoplasms with germline predisposition and pre-existing platelet disorder** |
| • Germline *RUNX1* P/LP variant (familial platelet disorder with associated myeloid malignancy, FPD-MM)<br>• Germline *ANKRD26* P/LP variant (Thrombocytopenia 2)<br>• Germline *ETV6* P/LP variant (Thrombocytopenia 5) |
| **Myeloid neoplasms with germline predisposition and potential organ dysfunction** |
| • Germline *GATA2* P/LP variant (*GATA2*-deficiency)<br>• Bone marrow failure syndromes<br>◦ Severe congenital neutropenia (SCN)<br>◦ Shwachman-Diamond syndrome (SDS)<br>◦ Fanconi anaemia (FA)<br>• Telomere biology disorders<br>• RASopathies (Neurofibromatosis type 1, CBL syndrome, Noonan syndrome or Noonan syndrome-like disorders<br>• Down syndrome<br>• Germline *SAMD9* P/LP variant (MIRAGE Syndrome)<br>• Germline *SAMD9L* P/LP variant (SAMD9L-related Ataxia Pancytopenia Syndrome<br>• Biallelic germline *BLM* P/LP variant (Bloom syndrome) |

## II. OBJECTIVES

The specific objectives of my PhD work were as follows:

- To perform a comprehensive genetic characterization of pediatric patients diagnosed with AML using panel-based targeted next-generation sequencing for the first time in a Hungarian patient cohort.
- To investigate the cytogenetic profiles of pediatric AML patients and the relationship between cytogenetic alterations and somatic mutations.
- To evaluate the clinical impact of the identified genetic alterations on event-free and overall survival.
- To provide a comparative analysis of genetic alterations detected in relapsed patients at the time of diagnosis and relapse.
- To evaluate the mutational landscape of inherited myeloid malignancies with the aim of uncovering genetic lesions contributing to disease development

## III. METHODS

### III.1. Patient samples

Seventy-five patients with pediatric AML were included in this study, with a female:male ratio of 1:1.2. The median age at diagnosis was 9.0 years (range, 0 to 17 years), and the median white blood cell count was $8.9x10^9$/L (range, 0.51 to $348x10^9$/L). Diagnostic bone marrow (n=70) or peripheral blood (n=2) samples from 72 children diagnosed with AML, as well as skin (n=1) or lymph node (n=2) samples from 3 children diagnosed with extramedullary AML, were analyzed. Diagnoses were established based on morphologic, immunophenotypical, and genotypical criteria at the Department of Pathology and Experimental Cancer Research, Semmelweis University, in the Department of Pathology, University of Pécs, or in the Department of Pathology, University of Debrecen, between 2003 and 2021 according to the classification system of WHO (154). Patients were risk stratified and treated according to Berlin-Frankfurt-Münster (BFM) protocols, including AML-BFM 98, AML-BFM 2004, AML-BFM 2012, and AML-BFM 2019 (Supplementary Table S1). In addition to the diagnostic samples of 75 patients, nine samples drawn at the time of the first relapse and three samples drawn at the second relapse were investigated. The median follow-up time was 23.8 months (range, 0.2 to 205.0 months). Additional clinical characteristics of patients are summarized in Supplementary Table S1. DNA was extracted from the corresponding specimens (bone marrow, n=79; peripheral blood, n=3; skin, n=1; lymph node, n=2; and cerebrospinal fluid, n=1) using the High Pure PCR Template Preparation *KIT* (Roche Life Science, Indianapolis, IN). Extracted DNA was quantified by using the Qubit dsDNA HS assay *KIT* and Qubit 4.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA). The study was approved by the Ethics Committee of the Hungarian Medical Research Council (IV/51-1/2022/EKU). Written informed consent from the patients and/or from the parents or guardians were obtained for the study, which was conducted in accordance with the Declaration of Helsinki.

In the frame of an international collaboration, DNA samples from well-characterized MDS/AML families ≥2 members were diagnosed with a hematological disorder, of which ≥1 case was specified as MDS/AML were included in this study. Overall, DNA samples from 51 individuals from 33 families were available. Of the 51 individuals, 16 were

diagnosed with AML, 22 MDS, 1 thrombocytopenia (TCP), 7 bone marrow failure (BMF), 1 lymphoedema, 1 had abnormal lymphocyte subsets, and 3 were asymptomatic carriers. Each individual carried a germline variant in 1 of 13 discrete loci known to predispose to familial MDS/AML (158). The median age of onset was 30 years (range, 1–76 years). The age of onset of MDS/AML was highly variable and dependent on the nature of the germline mutations. *RUNX1*, *CEBPA*, and *GATA2* families exhibited early-onset MDS/AML, with a median age of 10, 18, and 21 years at presentation, respectively, compared with *TERT*/TERC, *SRP72*, and *DDX41* families who had a longer latency and a median age of 41.5, 51 and 56 years, respectively.

### III.2. Cytogenetic analysis

Cytogenetic aberrations were determined by conventional G-banding and FISH to detect abnormalities related to AML. Probes for t(8;21)(q22;q22) *RUNX1::RUNX1T1* (ZytoVision, Bremerhaven, Germany), inv(16)(p13.1q22)/t(16;16) (p13.1;q22) *CBFB::MYH11* (Abbott Molecular, Des Plaines, IL); t(15;17)(q22;q12) *PML::RARA* (ZytoVision), and 11q23 *KMT2A* rearrangements (ZytoVision) were used for FISH analysis. A complex karyotype was defined as three or more chromosomal aberrations in the absence of the recurrent AML genetic aberrations defined by using the World Health Organization classification, including t(8;21)(q22;q22), inv(16)(p13.1q22)/t(16;16)(p13.1;q22), t(15;17)(q22;q12), t(6;9)(p23;q34.1), *KMT2A*/11q23 rearrangement, or t(9;22)(q34;q11.2). Patients with CBF-AML characterized by inv(16)(p13;1q22), t(16;16)(p13;q22), t(8;21)(q22;q22), and t(15;17) (q24;q21) were categorized in the favorable risk group; complex karyotype, monosomy 7, t(6;9)(p23;q34.1), inv(3) (q21q26), t(4;11)(q21;q23), t(6;11)(q27;23), and t(10;11) (p12;q23) were considered as adverse prognostic markers. Patients with normal karyotype and cytogenetic abnormalities not included in the aforementioned subgroups were classified into the intermediate-risk group.

### III.3. Assessment of *FLT3*-ITD and *CEBPA* Mutational Status

Mutation analysis of *FLT3*-ITD was performed from genomic DNA using primers adapted from Kottaridis et al. (11F: 5'-FAM-GCAATTTAGGTATGAAAGCCAGC-3'; 12R: 5'-CTTTCAGCATTTTGACGGCAACC-3') (159). Fluorescently labeled PCR

products were analyzed using a capillary electrophoresis 3500 Genetic Analyzer (Thermo Fisher Scientific) and GeneMapper software 5 (Thermo Fisher Scientific). The mutant *FLT3*-ITD allelic burden was calculated as the ratio of area under the mutant versus wild-type *FLT3* peak. For the assessment of the mutation status of *CEBPA*, the entire coding sequence was amplified by using three overlapping PCR fragments with previously described primer pairs and analyzed by bidirectional Sanger sequencing (160).

### III.4. Targeted next-generation sequencing and bioinformatic analysis

Targeted NGS was performed using the TruSight Myeloid Sequencing Panel (Illumina, San Diego, CA) covering 54 leukemia-associated genes (Table 3). Individual libraries were prepared from 50 ng of genomic DNA according to the manufacturer's recommendations. DNA extracted from peripheral blood mononuclear cells of 15 healthy volunteers with normal complete blood profiles was used as negative control subjects. After quality control and equimolar pooling, libraries were sequenced on a NextSeq 550 platform (Illumina) using v2.5 chemistry with 150 bp paired-end configuration. For the pediatric AML cohort, raw sequencing data generated from TruSight Myeloid libraries were analyzed by using the TruSeq Amplicon app in BaseSpace Sequence Hub (Illumina). After demultiplexing and FASTQ file generation, reads were aligned against the GRCh37 reference human genome with a custom banded Smith-Waterman aligner. Single-nucleotide polymorphisms and short insertions or deletions were identified using the Genome Analysis Toolkit (Broad Institute, Cambridge, MA; https://gatk.broadinstitute.org).

Variants were further processed by using a custom Snakemake pipeline. SnpSift version 4.3t (https://pcingola.github.io/SnpEff) was used for annotating variants with dbSNP version 20180423 (https://www.ncbi.nlm.nih.gov/snp), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar, last accessed July 6, 2020), or COSMIC version 92 (https://cancer.sanger.ac.uk/cosmic) coding mutations. In addition, ENSEMBL VEP (https://www.ensembl.org/info/docs/tools/vep/index. html, last accessed June 26, 2020; annotation data set downloaded at the same time) was used for annotating variant consequences or impact, and allele frequency data from the 1000 Genomes, gnomAD, or NHLBI GO Exome Sequencing Project (ESP). For reliable detection of high-confidence mutations, variants were filtered based on several criteria: for each sample, variants were

excluded if coverage was <100 reads, <20 reads supported the variant allele, or the variant allele frequency (VAF) was <5%. Synonymous variants and known single-nucleotide polymorphisms were excluded (based on an overall population allele frequency of >1% according to the gnomAD database).

**Table 3.** List of genomic regions captured by the Illumina TruSight Myeloid Sequencing Panel.

| Gene | Targeted Region (exon) | Gene | Targeted Region (exon) |
|---|---|---|---|
| *ABL1* | 4–6 | *JAK3* | 13 |
| *ASXL1* | 12 | *KDM6A* | full CDS |
| *ATRX* | 8–10,17–31 | *KIT* | 2,8–11,13,17 |
| *BCOR* | full CDS | *KRAS* | 2,3 |
| *BCORL1* | full CDS | *KMT2A (MLL)* | 5–8 |
| *BRAF* | 15 | *MPL* | 10 |
| *CALR* | 9 | *MYD88* | 3–5 |
| *CBL* | 8,9 | *NOTCH1* | 26-28,34 |
| *CBLB* | 9,1 | *NPM1* | 12 |
| *CBLC* | 9,1 | *NRAS* | 2,3 |
| *CDKN2A* | full CDS | *PDGFRA* | 12,14,18 |
| *CEBPA* | full CDS | *PHF6* | full CDS |
| *CSF3R* | 14–17 | *PTEN* | 5,7 |
| *CUX1* | full CDS | *PTPN11* | 3,13 |
| *DNMT3A* | full CDS | *RAD21* | full CDS |
| *ETV6/TEL* | full CDS | *RUNX1* | full CDS |
| *EZH2* | full CDS | *SETBP1* | 4 (partial) |
| *FBXW7* | 9–11 | *SF3B1* | 13–16 |
| *FLT3* | 14,15,20 | *SMC1A* | 2,11,16,17 |
| *GATA1* | 2 | *SMC3* | 10,13,19,23,25,28 |
| *GATA2* | 2–6 | *SRSF2* | 1 |
| *GNAS* | 8,9 | *STAG2* | full CDS |
| *HRAS* | 2,3 | *TET2* | 3–11 |
| *IDH1* | 4 | *TP53* | 2–11 |
| *IDH2* | 4 | *U2AF1* | 2,6 |
| *IKZF1* | full CDS | *WT1* | 7,9 |
| *JAK2* | 12,14 | *ZRSR2* | full CDS |

The variants were filtered according to different criteria in the cohort of patients with inherited myeloid malignancies. All variants in the 1000 Genomes project (1000G) or NHLBI GO-ESP with a frequency of >0.5% were removed and retained non-synonymous exonic or splicing calls with a variant allele frequency greater than 5%. Variants that

occurred in 10 or more samples or those with a sequencing depth less than 20 were removed. The mean sequencing depth was 1300 reads/locus.

### III.5. Droplet Digital PCR

Screening and quantitative assessment of the *FLT3* p.D835Y mutation were performed by ddPCR using a mutation-specific assay for *FLT3* p.D835Y (assay ID: dHsaMDV2010047; Bio-Rad Laboratories, Hercules, CA). Reactions were performed according to the manufacturer's recommendations. Droplets were generated by using the QX200 Automated Droplet Generator (Bio-Rad Laboratories) followed by fluorescent signal detection with the QX200 Droplet Reader system (Bio-Rad Laboratories). Results were evaluated and quantified by using QuantaSoft software version 1.7 (Bio-Rad Laboratories). The allelic burden of the mutation was defined as fractional abundance calculated from the ratio of the number of mutant DNA molecules (a) and the total number of mutant (a) plus wild-type (b) DNA molecules detected: fractional abundance Z a/(a + b). The sensitivity of the ddPCR analysis was assessed for each sample; the lower limit of the quantitative range could ubiquitously be determined as 0.01%.

### III.6. Statistical Methods

EFS and OS were estimated by using the Kaplan-Meier method and compared statistically by using the log-rank test. CR was defined as <5% blasts in the bone marrow, no evidence of leukemia at any other site, and evidence of regeneration of normal hematopoietic cells. OS was calculated from the date of diagnosis to exit or last follow-up. EFS was calculated from the date of diagnosis to the first event (induction failure, relapse, or death) or to the date of the last follow-up (death from early toxicity was excluded). Patients who failed to achieve CR on day 60 were considered as treatment failures.

# IV. RESULTS

## IV.1. Genomic characterization of patients with pediatric AML

### IV.1.1. Cytogenetic profiles of pediatric AML patients at diagnosis

Cytogenetic analysis is essential for the classification and prognostication of AML; therefore, it was performed as part of the standard-of-care diagnostic workup. Cytogenetic results were available in 71 out of 75 patients with normal karyotype detected in 22.5% (n=16) of the patients. *KMT2A*-rearrangements were the most frequently observed (n=16, 22.5%) cytogenetic aberrations, followed by CBF-rearrangements (n=10, 14.1%): t(8;21) translocations (n=7) and inv(16)/t(16;16) alterations (n=3). Eleven patients had karyotypes associated with adverse prognosis (complex karyotype, monosomy 7, t(6;9) or inv(3)). Twenty percent of the patients (n=15) were characterized by 'other' aberrations (i.e. cytogenetic abnormalities not classified in the above-mentioned subgroups) (Figure 2). The cytogenetic categories showed age-related distribution with younger children harboring *KMT2A*-rearrangement and 'other' aberrations more frequently, while the abundance of CBF-rearrangements and adverse karyotype increased with age (Figure 3).

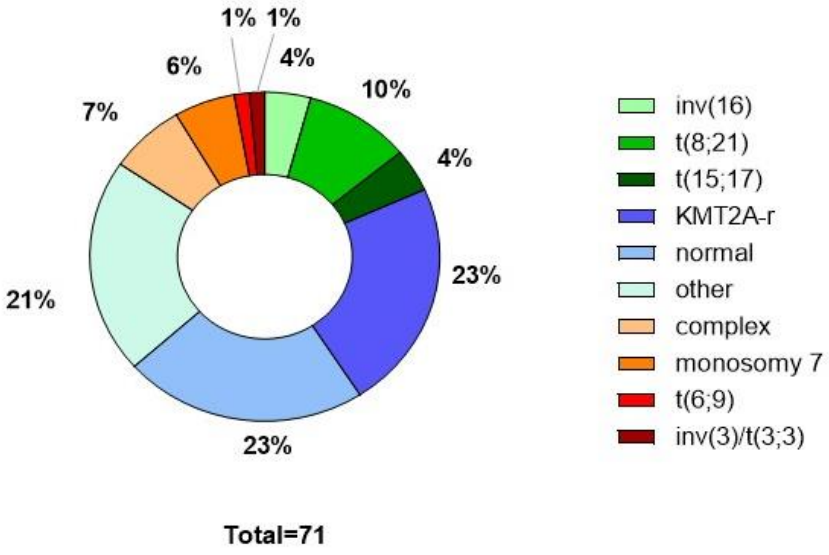



**Figure 2.** Pie chart showing the frequency of the major cytogenetic subgroups in pediatric AML. Favorable risk cytogenetic groups are shown in shades of green, the poor risk groups in shades of red, while shades of blue indicate intermediate risk subgroups.

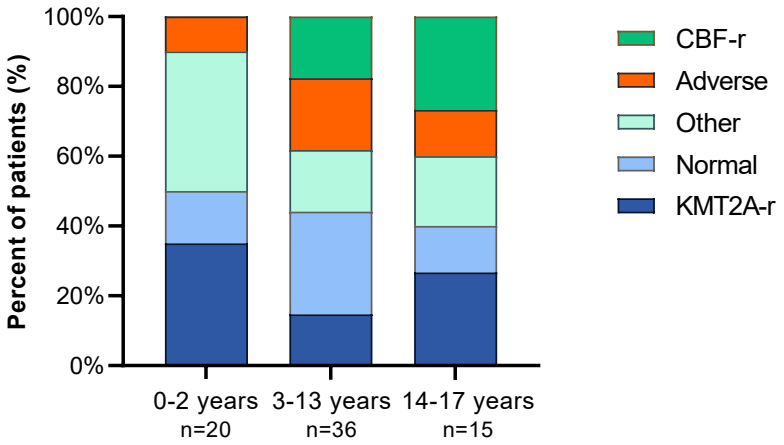



**Figure 3.** Distribution of the cytogenetic subgroups according to age groups. Cytogenetics appeared different according to age groups with younger children harboring *KMT2A*-rearragement and 'other' aberrations more frequently, while the ratio of CBF-rearrangement increased with age.

### IV.1.2. Targeted NGS-based mutation profiling of pediatric AML at diagnosis

Targeted NGS with an average allelic depth of 4,960x revealed in total 154 single nucleotide variants (SNVs) and short insertions/deletions in the diagnostic samples of 74 patients. The number of mutations identified in patients at diagnosis showed uneven distribution across specific cytogenetic subgroups, although these differences were not statistically significant (Figure 2). Median number of mutations per patient was 2.0 (range: 0-18) with the highest rate of mutations in CN-AML (3.0) and the lowest in *KMT2A*-rearranged AML (1.0) (Figure 4). In terms of mutation classes, missense and frameshift mutations were the most commonly detected, followed by duplications and splice site variants (Figure 5-6).

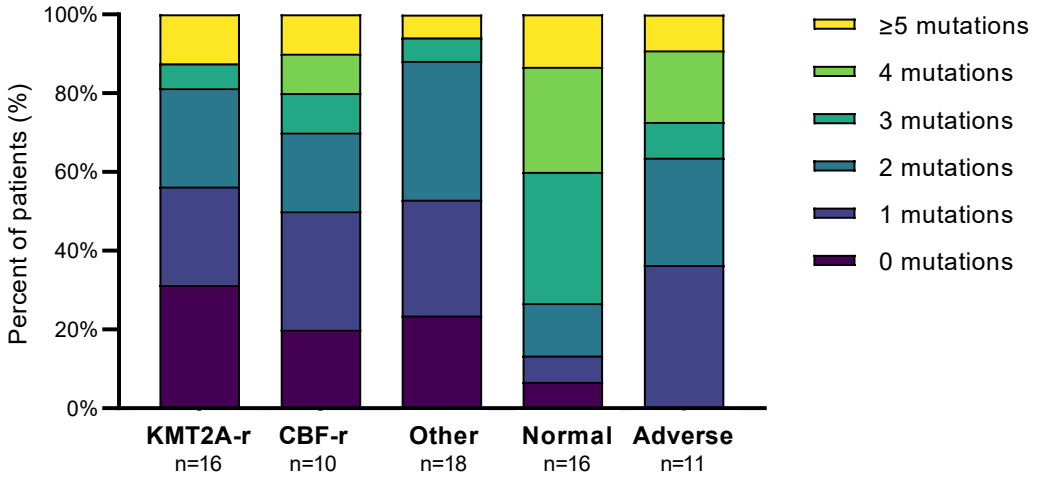



**Figure 4.** Number of mutations detected at diagnosis according to cytogenetic subgroup. The highest mutation rate was detected in normal karyotype AML and karyotypes associated with adverse prognosis.

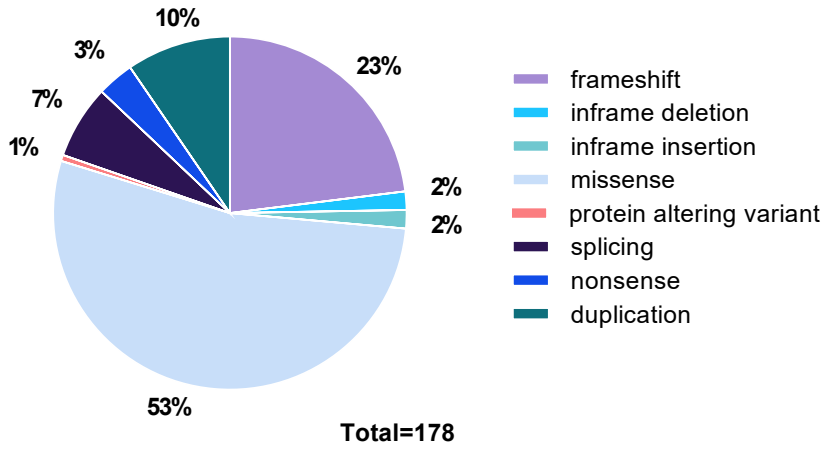



**Figure 5**. Pie chart showing the distribution of mutation types in the diagnostic samples of 74 pediatric AML patients. Frequencies are based on the results of NGS analysis (n=154), fragment analysis of *FLT3*-ITD (n=17) and Sanger sequencing of *CEBPA* (n=7).

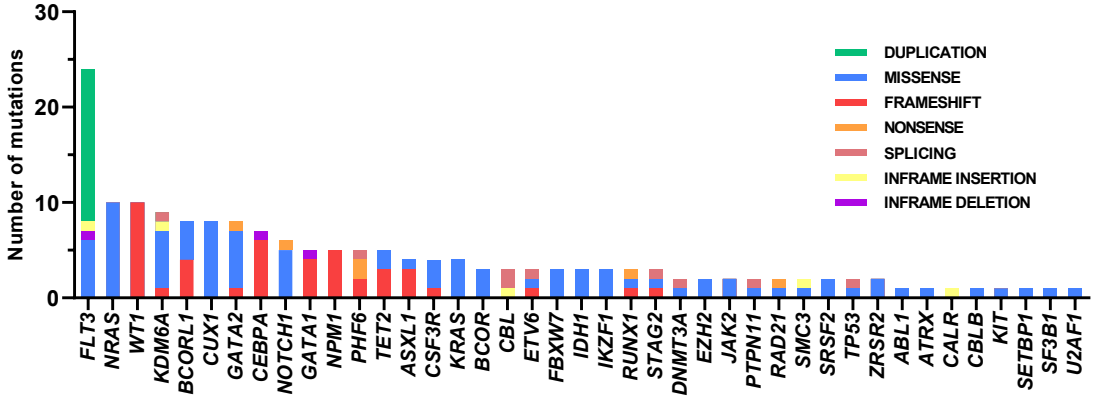



**Figure 6.** Bar graph depicting the total number of mutations detected in individual genes ranked in order of recurrence. Different colors distinguish between mutation types.

Distribution of VAFs showed heterogeneity with most genes being affected by both subclonal and clonal alterations. Mutations in *ASXL1*, *CBL*, *ETV6*, *IDH1* and *NPM1* emerged with a VAF of >30% in all cases (Figure 7). Overall, 83.8% of patients (62/74) carried at least one mutation in genes analyzed by NGS. Considering all genetic alterations detected by different modalities including cytogenetics, aberrations were identified in 98.6% (73/74) of patients.

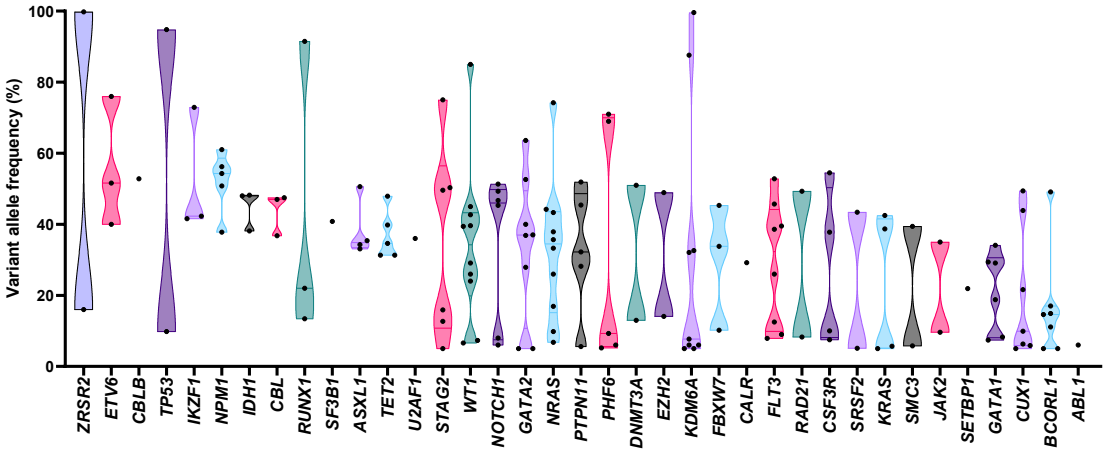



**Figure 7.** Variant allele frequencies in individual genes across the 40 genes analyzed by NGS.

The most common class of mutations involved genes controlling kinase signaling (36.7%; n=65) and encoding transcription factors (20.9%; n=37), followed by chromatin modifiers (15.3%; n=27), tumor suppressors (9.6%; n=17), DNA methylation (5.6%;

n=10), cohesion genes (5.6%; n=10) and RNA splicing (3.4%; n=6). Figure 8A depicts the pairwise co-occurrence of the mutations in the different functional subgroups. Mutations associated with activated signaling commonly emerged together and with mutations of genes encoding transcription factors and epigenetic modifiers, while *NPM1* and epigenetic modifier mutations were mutually exclusive (Figure 8B). Mutations of kinase signaling genes were found in 60.8% of patients spread across all subtypes.

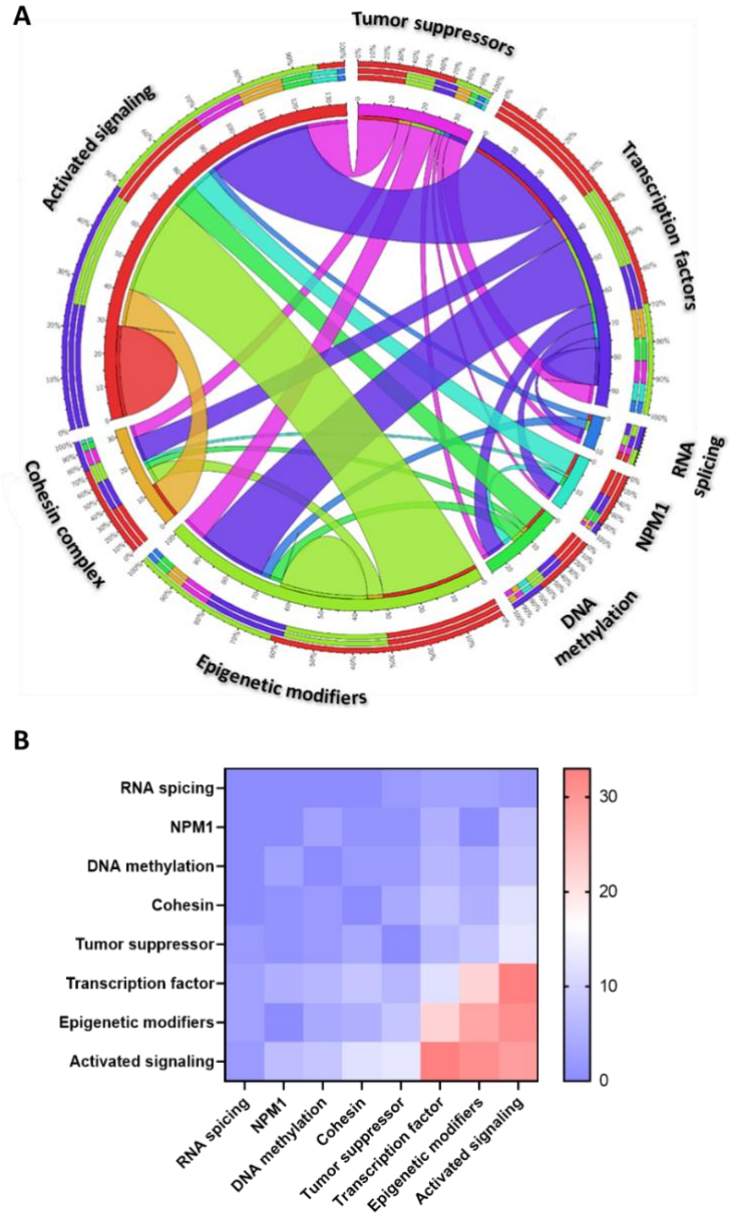



**Figure 8.** (A) Circos plot diagram illustrating the pairwise co-occurrence of molecular aberrations based on the functional groups. (B) Mutations associated with activated signaling commonly emerged together and with mutations of genes encoding transcription factors and epigenetic

modifiers. Mutations of tumor suppressor genes occurred concomitantly with mutations of other functional groups.

Of the 54 genes examined, 40 genes were altered in our cohort, with 17 genes recurrently mutated in over 5% of patients (Figure 9). *FLT3* (24%, 18/74), *NRAS* (14%, 10/74) and *GATA2* (11%, 8/74) represented the most frequently mutated genes (Figure 9-10). *FLT3*-ITDs were detected in 14.9% (11/70) of the diagnostic patient samples, including a total of 17 *FLT3*-ITD mutations with median allelic ratio of 0.09 (range: 0.02 - 4.91). At the same time, the length of ITD varied between 6 and 96 base pairs (Table 4). The majority (63.6%; 7/11) of *FLT3*-ITD+ cases carried a single mutation, while 18.2% (2/11) of patients harbored two, and 18.2% (2/11) three mutations. As expected, *FLT3*-ITD mutations were predominantly present in patients with CN-AML (27%; 4/15) and CBF-AML (20%; 2/10).

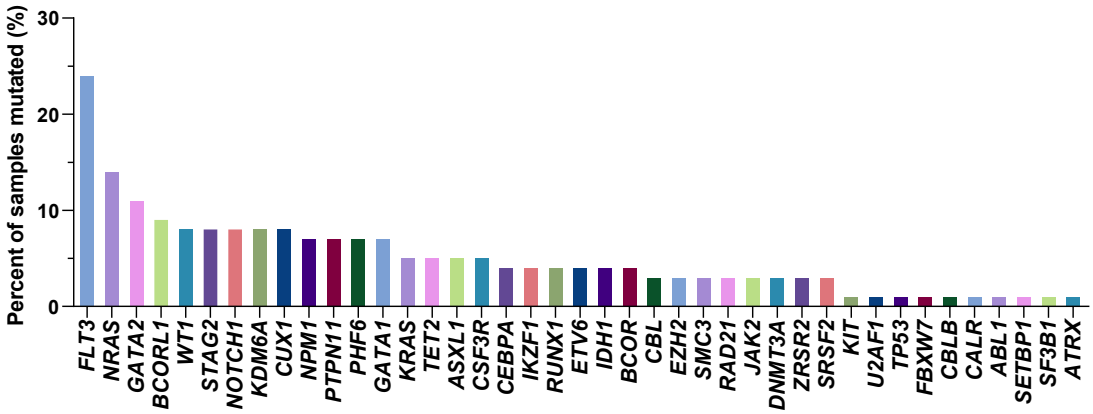
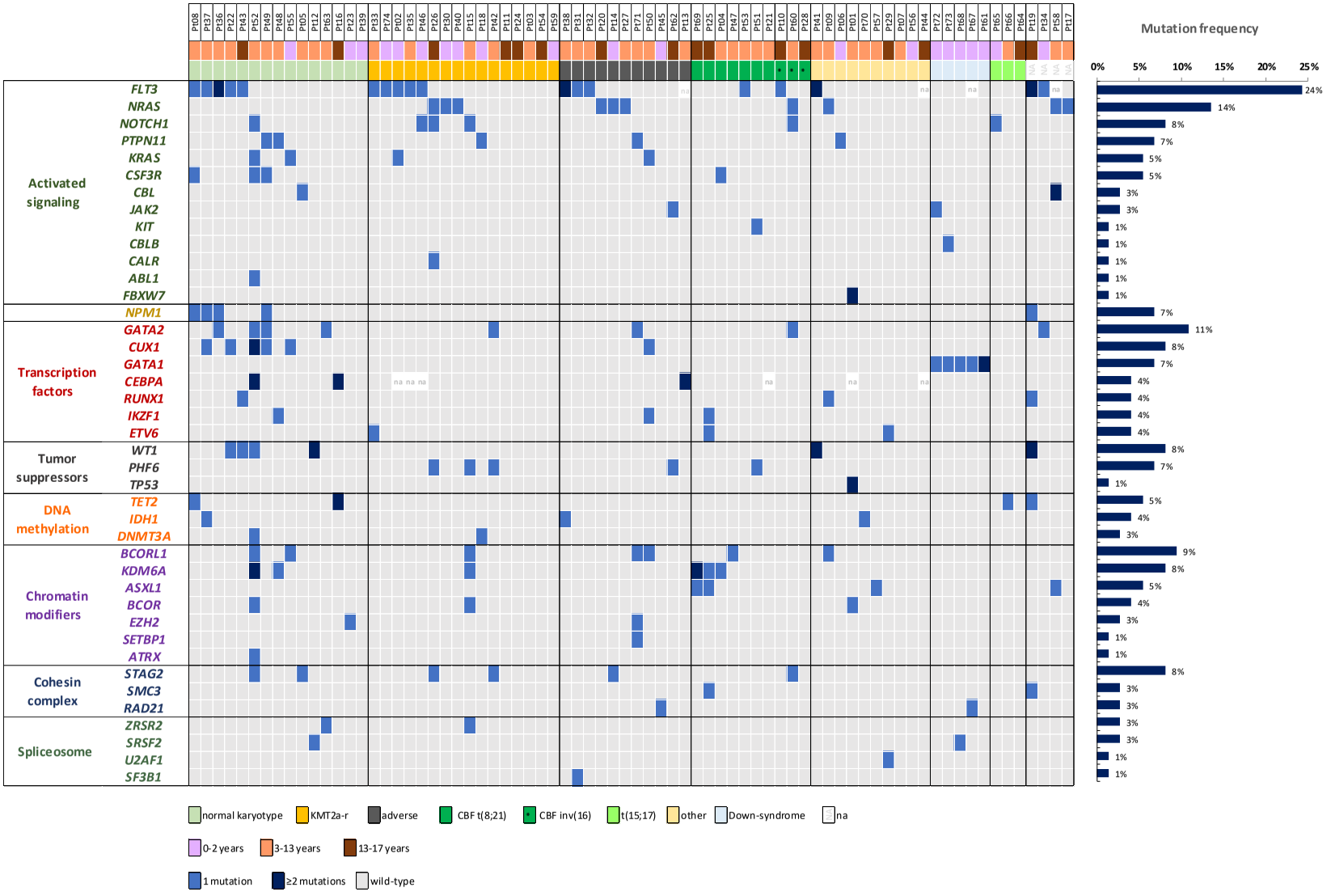



**Figure 9.** Bar plot showing mutations ranked by frequency of mutations in patients.

Following *FLT3*-ITD mutations, alterations of the TKD of *FLT3* were the most frequently observed, as *FLT3*-TKD mutations were present in 8.1% (6/74) of patients: mutations occurred in codons 835 (n=4), 836 (n=1) and 841 (n=1). Of the six patients with *FLT3*-TKD mutation, 4 patients had *KMT2A*-rearrangement. Conversely, 31.0% (5/16) of patients with *KMT2A*-rearrangements harbored *FLT3* mutation, and interestingly, no *FLT3*-ITD, only *FLT3*-TKD mutation was found in this subgroup. Additionally, a single patient harbored a point mutation at the juxtamembrane domain of *FLT3*.

**Table 4**. *FLT3*-ITD mutations detected by fragment analysis.

| Table | Diagnosis / Relapse | Number of *FLT3*-ITD mutations per sample | *FLT3*-ITD length (bp) | Allelic ratio (*FLT3*-TD/*FLT3*wt) |
|---|---|---|---|---|
| P10 | Diagnosis | 1 | 51 | 0.09 |
| P19 | Diagnosis | 2 | 21 | 1.57 |
| P19 | Diagnosis | 2 | 51 | 6.25 |
| P22 | Diagnosis | 1 | 30 | 0.02 |
| P31 | Diagnosis | 1 | 36 | 4.68 |
| P31 | Relapse1 | 1 | 36 | 0.59 |
| P31 | Relapse2 | 1 | 36 | 12.04 |
| P34 | Diagnosis | 1 | 87 | 0.44 |
| P36 | Diagnosis | 2 | 30 | 0.02 |
| P36 | Diagnosis | 2 | 75 | 0.17 |
| P37 | Diagnosis | 1 | 39 | 0.78 |
| P38 | Diagnosis | 3 | 21 | 0.02 |
| P38 | Diagnosis | 3 | 30 | 0.02 |
| P38 | Diagnosis | 3 | 48 | 0.57 |
| P41 | Diagnosis | 3 | 45 | 0.08 |
| P41 | Diagnosis | 3 | 57 | 0.31 |
| P41 | Diagnosis | 3 | 69 | 0.02 |
| P43 | Diagnosis | 1 | 96 | 0.09 |
| P53 | Diagnosis | 1 | 6 | 0.02 |


Mutation frequency
0%
5%
10%
15%
20%
25%
Activated signaling
FLT3
NRAS
NOTCH1
PTPN11
KRAS
CSF3R
CBL
JAK2
KIT
CBLB
CALR
ABL1
FBXW7
NPM1
Transcription factors
GATA2
CUX1
GATA1
CEBPA
RUNX1
IKZF1
ETV6
Tumor suppressors
WT1
PHF6
TP53
DNA methylation
TET2
IDH1
DNMT3A
Chromatin modifiers
BCORL1
KDM6A
ASXL1
BCOR
EZH2
SETBP1
ATRX
Cohesin complex
STAG2
SMC3
RAD21
Spliceosome
ZRSR2
SRSF2
U2AF1
SF3B1
24%
14%
8%
7%
5%
5%
3%
3%
1%
1%
1%
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1%
8%
3%
3%
3%
3%
1%
1%
normal karyotype
KMT2a-r
adverse
CBF t(8;21)
CBF inv(16)
t(15;17)
other
Down-syndrome
na
0-2 years
3-13 years
13-17 years
1 mutation
≥2 mutations
wild-type

**Figure 10. Heat map displaying the somatic variants detected in the 54 target genes analyzed in the diagnostic samples of 74 pediatric AML patients**. Illustrated is the distribution of the somatic variants, age groups, and cytogenetic profiles as determined by karyotyping or FISH, as well as the mutation frequency of the individual genes for all cases.

RAS pathway mutations (*NRAS*, *KRAS*, *PTPN11*, *CBL*) were present in 27.0% (20/74) of patients and mainly occurred in *KMT2A*-rearranged cases and CN-AML. Most *NRAS* mutations (7/10) were found at codon 61, while mutations at codons 12 and 13 were detected in 2 and 1 case, respectively. The median VAF of *NRAS* mutations was 35% (7-74%).

*NPM1* mutations were detected in 6.8% (5/74) of patients. Of the 5 mutations, 4 have been previously reported: 3 patients had type A mutation (c.863_864dupTCTG), 1 patient had type J mutation (c.863_864insCCGG), and one patient presented with a novel *NPM1* mutation (c.869_873delinsCCCTTTCCC). Four out of 5 patients with *NPM1* mutant harbored concomitant *FLT3* mutations. *NPM1* mutation was associated with normal karyotype (p=0.0015), in line with previous publications.

*KDM6A* mutations were present in 8.1% of patients (6/74) at diagnosis with close association with *CBF* rearrangements, as 30% (3/10) of patients with CBF-AML harbored *KDM6A* mutations (p=0.0343). Notably, *KDM6A* mutations were restricted to patients with t(8;21) AML, while they were absent in inv(16)/t(16;16)). Regarding *CBF*-rearranged AML, no enrichment of *KIT* or *RAS* mutations was found in this subgroup of our cohort.

Besides genes with known mutational hotspots (*NRAS*, *FLT3*, *IDH1*, *ASXL1*, *SRSF2*) recurrent variants were detected in *CUX1* and *BCORL1* genes. *CUX1* c.1573C>G p.Leu525Val and c.1613A>G p.Asp538Gly variants were detected in 2-2 patients, respectively, with VAFs ranging from 6% to 44%. Overall, *CUX1* mutations were detected in 8% (6/74) of patients, predominantly associated with CN-AML (5/6; p=0.0013). Nine percent (7/74) of patients carried *BCORL1* mutations, including a recurrent frameshift mutation (c.2541del p.Ser848ValfsTer5) identified in 3 patients previously not described.

The entire coding region of *CEBPA* gene was screened in 68 patients. *CEBPA* mutations were detected in 3 patients (4.4%), all of whom harbored both N- and C-terminal type of mutations. Two novel frameshift mutations were identified (c.950_953delinsACCTT p.Leu317HisfsTer4; c.691_701del p.Val232AlafsTer85) in our cohort. Out of the three *CEBPA* mutant children two had de novo AML with normal karyotype, and one patient presented with therapy-associated AML with complex karyotype.

### IV.1.3. Comparative analysis of mutation profiles at diagnosis and relapse

To uncover progression related changes in mutational profile, targeted NGS analysis was performed on matched diagnosis-relapse samples of 8 patients with pediatric AML. Overall, 9 first relapse samples and 3 second relapse samples were analyzed (in a single case no diagnostic DNA sample was available). The relapse samples carried a slightly higher number of mutations compared to the diagnostic samples, with an average of 2.5 mutations (range: 1-6) per sample in the relapsed cohort (vs 2.0 at diagnosis). 61.5% (8/13) of initially detected mutations persisted at relapse, 38.5% (5/13) of mutations were detected only in the diagnostic sample, while 65.4% of mutations (17/26 relapse mutation) emerged during disease progression. Mutations that persisted from diagnosis to relapse had a higher VAF at diagnosis compared with those that were eliminated at relapse (median VAF at diagnosis: 30.7% vs 10.9%). However, due to the relatively limited number of samples analyzed, this did not reach statistical significance. At relapse, mutations were identified in 16 genes with *WT1* (42%; 5/12), *FLT3* (42%; 5/12), *NRAS* (33%; 4/12) and *NPM1* (25%; 3/12) representing the top four affected genes (Figure 11). Comparison of the function of the affected genes at diagnosis and relapse unveiled enrichment of mutations of genes affecting tumor suppression (44.4% vs. 16.2%) and transcription factors (55.6% vs. 35.1%) at relapse.

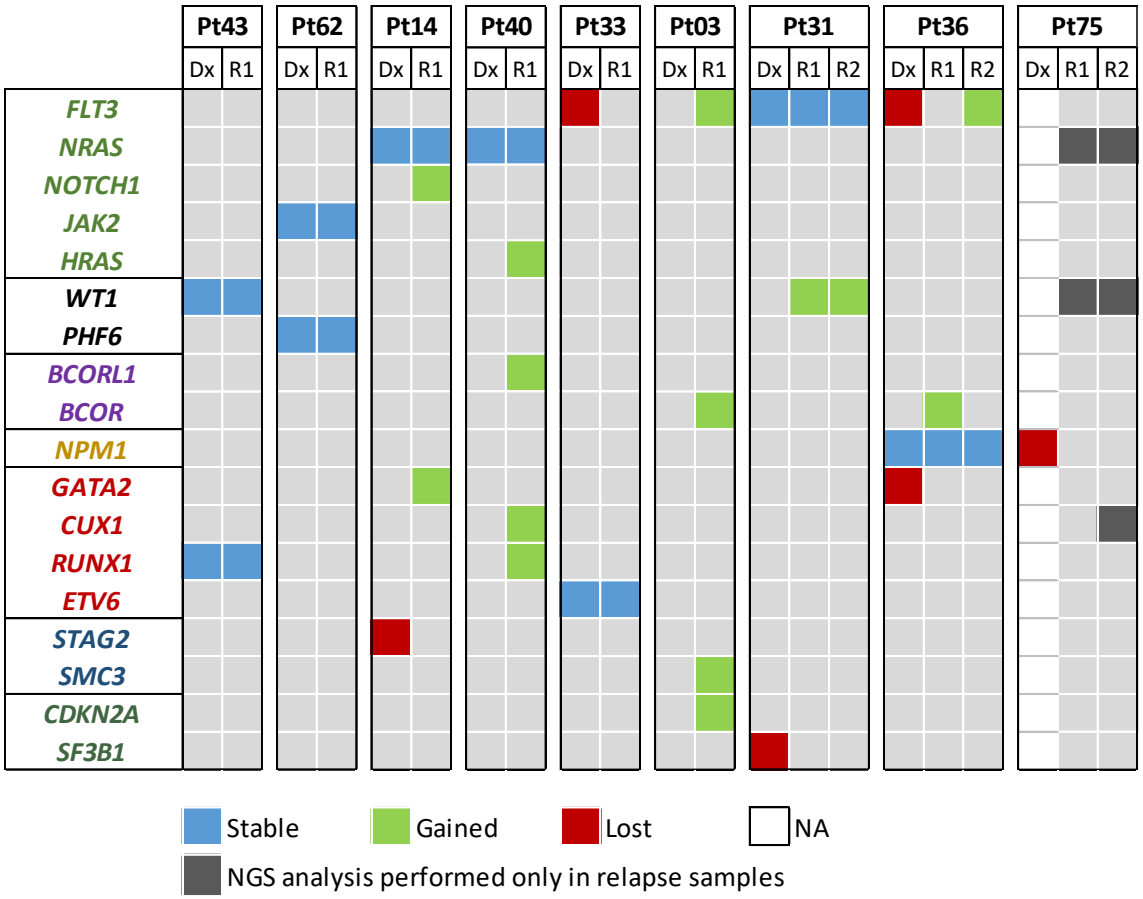


**Figure 11.** Heat map displaying the mutational status of nine patients at the time of diagnosis and relapse. Stable mutations (i.e. present at diagnosis and relapse) and unstable mutations (i.e. present either only at diagnosis or at relapse) are shown with different colors. (DNA was not available from Patient #75 to perform NGS analysis at diagnosis, however, the *NPM1* mutational status was known.)

Comparison of matching mutation profiles between clones dominating at diagnosis and relapse revealed three different patterns: (i) completely identical mutational profiles were observed in two patients (Patient #43, Patient #62) at diagnosis and relapse; (ii) in five patients the founding clone in the primary tumor gained additional mutations and evolved into the relapsed clone; (iii) while in a single patient the mutation profile slightly changed from diagnosis to relapse, as the initially detected *FLT3*-TKD mutated subclone was eradicated by the chemotherapy (Figure 11).

Patient #75 relapsed >3 years following initial diagnosis and presented with an entirely different leukemic clone regarding not only the mutation profile but also the immunophenotype and the karyotype (Figure 12). At diagnosis, this patient presented with CN-AML with *NPM1* mutation (no DNA sample was available for targeted NGS), while at first relapse, the *NPM1* mutation was eradicated, and in the meantime, monosomy 7, *NRAS,* and *WT1* mutations emerged, raising the possibility that instead of a clonally related relapse, the patient developed therapy-related AML. Following allogeneic hematopoietic stem cell transplantation (alloHSCT) the patient experienced a second relapse from the clone detected at the first relapse carrying monosomy 7 with a newly acquired subclonal *CUX1* mutation (the presence of *NRAS*, *WT1* and *CUX1* variants in the diagnostic sample could not be tested due to lack of DNA sample for targeted NGS).

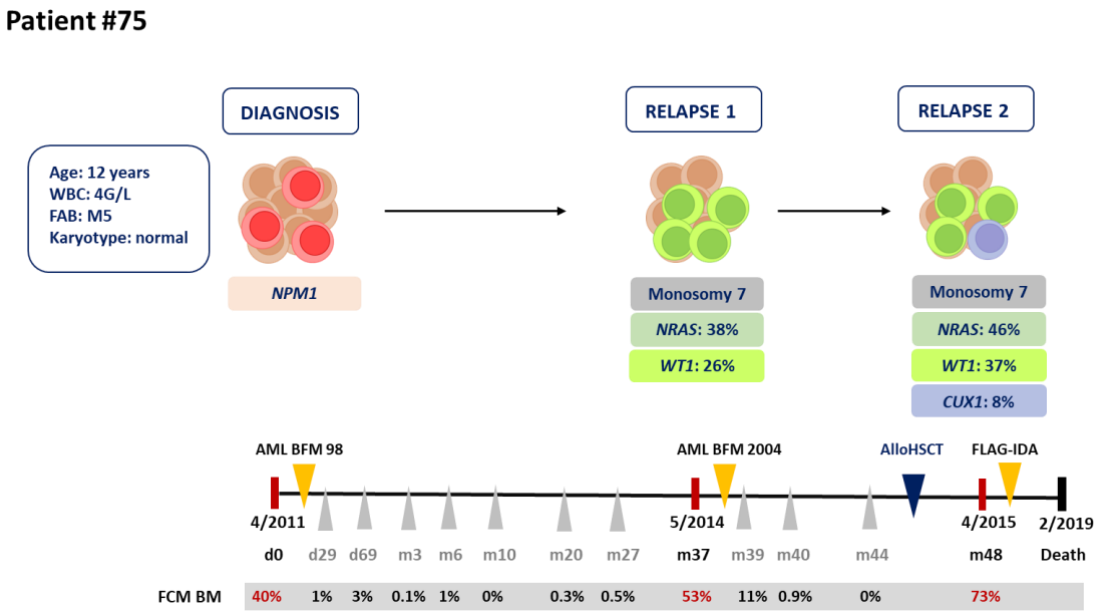


**Figure 12.** Detailed illustration of the clinical and genetic events from diagnosis to relapse. Comparison of matching mutation profiles between clones dominating at diagnosis and relapse revealed emergence of an entirely different leukemic clone.

Except for two patients (Patient #40 and Patient #3) who relapsed very early and showed active clonal evolution, time from diagnosis until the first relapse suggested a prolonged time requirement of clonal evolution from a founding clone compared to the quick return of an identical clone at the time of relapse (median 24.5 vs 9.5 months. To gain further

insights into the clonal dynamics, we studied Pt36, who had samples available from multiple disease time points. The patient initially presented with *NPM1*, *FLT3*-ITD and *GATA2* mutations at diagnosis; 2 years later she eventually relapsed with a clone carrying the original *NPM1* mutation and a newly acquired *BCOR* mutation, and shortly after achieving CR a second relapse evolved with the same *NPM1* mutation accompanied by a novel *FLT3* D835Y mutation. Interestingly, *FLT3* D835Y mutation was detectable by ddPCR with a low VAF (1.67%) as early as the time of the first relapse. The mutation was still present when the patient achieved CR2 with MRD positivity after two courses of salvage chemotherapy (VAF: 0.19%) (Figure 13).

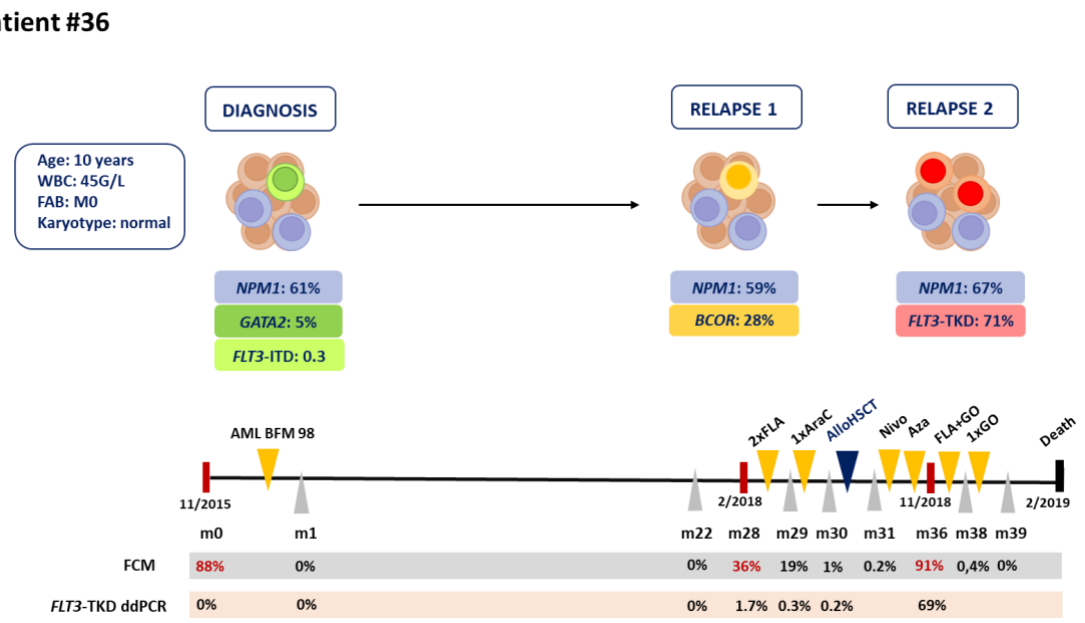


**Figure 13.** Detailed illustration of the clinical and genetic events from diagnosis to relapse. Comparison of matching mutation profiles between clones dominating at diagnosis and relapse revealed branching evolution with the acquisition of additional mutations. *Abbreviations: allo-HSCT: allogeneic hematopoietic stem cell transplantation, AraC: cytarabine, Aza: azacitidine, BFM: Berlin-Frankfurt-Münster, ddPCR: droplet digital polymerase chain reaction, FAB: French-American-British, FCM: flow cytometry, FLA: fludarabine, GO: gemtuzumab ozogamicin, m:month, Nivo: nivolumab, TKD: tyrosine kinase domain, WBC: white blood cell*

### IV.1.4. Evaluation of clinical outcomes of patients based on molecular profiles

At 5 years, the EFS and OS for the whole cohort proved to be 50.0% and 56.2% (Figure 14), respectively, with a median follow-up time of 23.8 months (range: 0.2-205). Risk classification based on cytogenetics and mutational status of *NPM1*, *CEBPA*, *FLT3*-ITD and *WT1* revealed that patients in the favorable-, intermediate and adverse-risk categories had significantly different 5-year EFS and OS (EFS: 90% vs. 30% vs. 18%, p<0.0001; OS: 90% vs. 42% vs. 22%; p=0.0014) (Figure 15). Multiparametric flow cytometry MRD follow-up data was available from 61 patients. CR after 2 courses of intensive chemotherapy was reached by 91.8% (56/61) of patients, while three patients experienced fatal complications during the induction therapy, and two patients were non-responders.

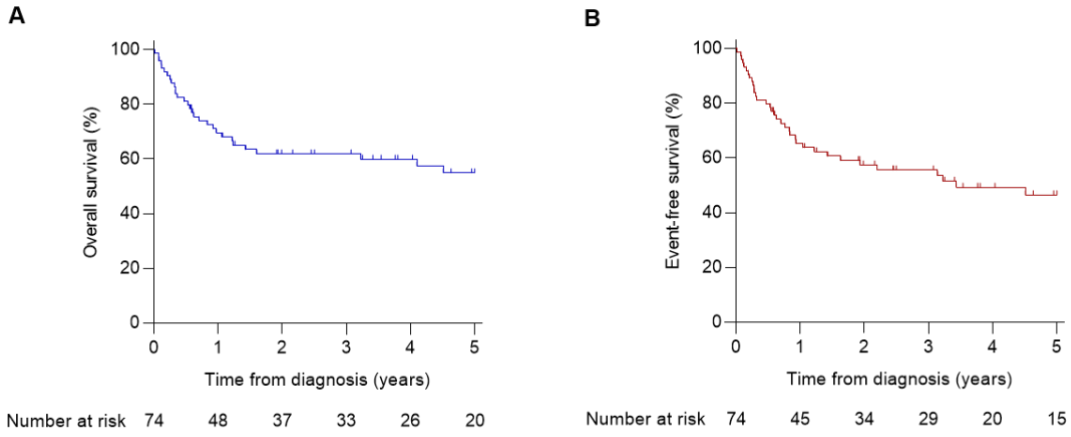


**Figure 14.** Five-year (a) event-free survival and (b) overall survival rates of the total cohort.

Due to the small number of patients, assessment of the prognostic significance of individual mutations was limited; only mutations in tumor suppressor genes (*TP53*, *PHF6,* and *WT1*) were significantly associated with induction failures (Fisher exact test: p=0.0039) and shorter three-year EFS (22.2% vs 61.2%; Fisher exact test: p=0.044) (Figure 15).

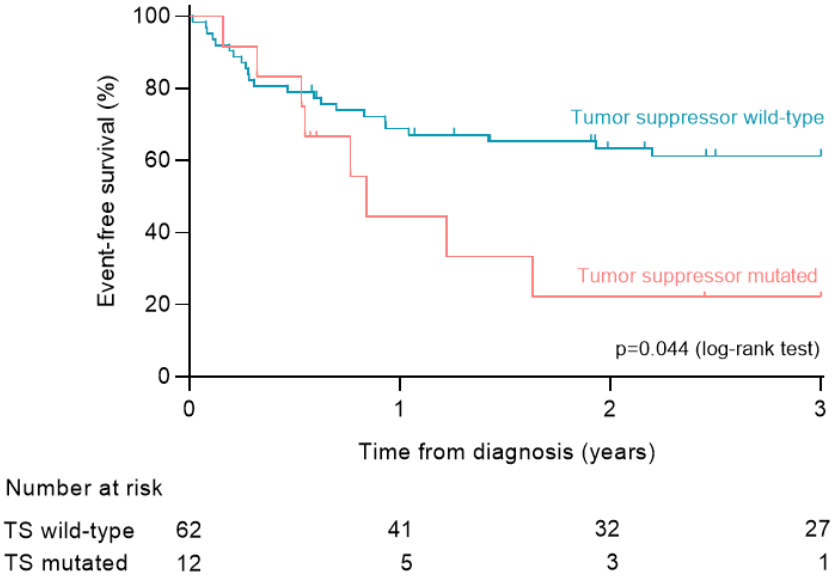


**Figure 15**. Three-year event-free survival of patients with wild-type tumor suppressor genes and with tumor suppressor mutations.

We investigated the prognostic significance of early response to treatment on day 28. AML BFM protocols define >20% blasts on day 28 as poor response to treatment. Using this cutoff value, both the 5-year EFS and OS significantly differed between good and poor responders (57.3% vs. 0%, p<0.0001; 64.2% vs. 28.6%, p=0.0414). We also investigated whether lowering the blast cutoff to 5% and assessing the MRD status at day 28 influence prognosis as evaluated by flow cytometry; however, interestingly, no difference was observed in the 5-year EFS and OS of patients (data not shown).

Stratification based on AML-BFM protocols was the following: patients with *RUNX1::RUNX1T1*, *CBFB::MYH11*, *PML::RARA,* normal karyotype with *NPM1* or biallelic *CEBPA* mutations were categorized in the favorable subgroup. Patients with complex karyotype (i.e. >3 aberrations), monosomy 7, t(6;9), inv(3), t(6;11), t(10;11) or *FLT3*-ITD with *WT1* mutation belonged to the adverse risk group, with all remaining patients categorized as intermediate risk. The 5-year OS was 90.0% for patients with favorable prognosis, 42.3% for patients with intermediate risk and 22.2% among patients with unfavorable prognosis (p=0.0014), while the EFS values in these risk groups proved to be 90.0%, 30.0 and 18.5%, respectively (p<0.0001) (Figure 16).

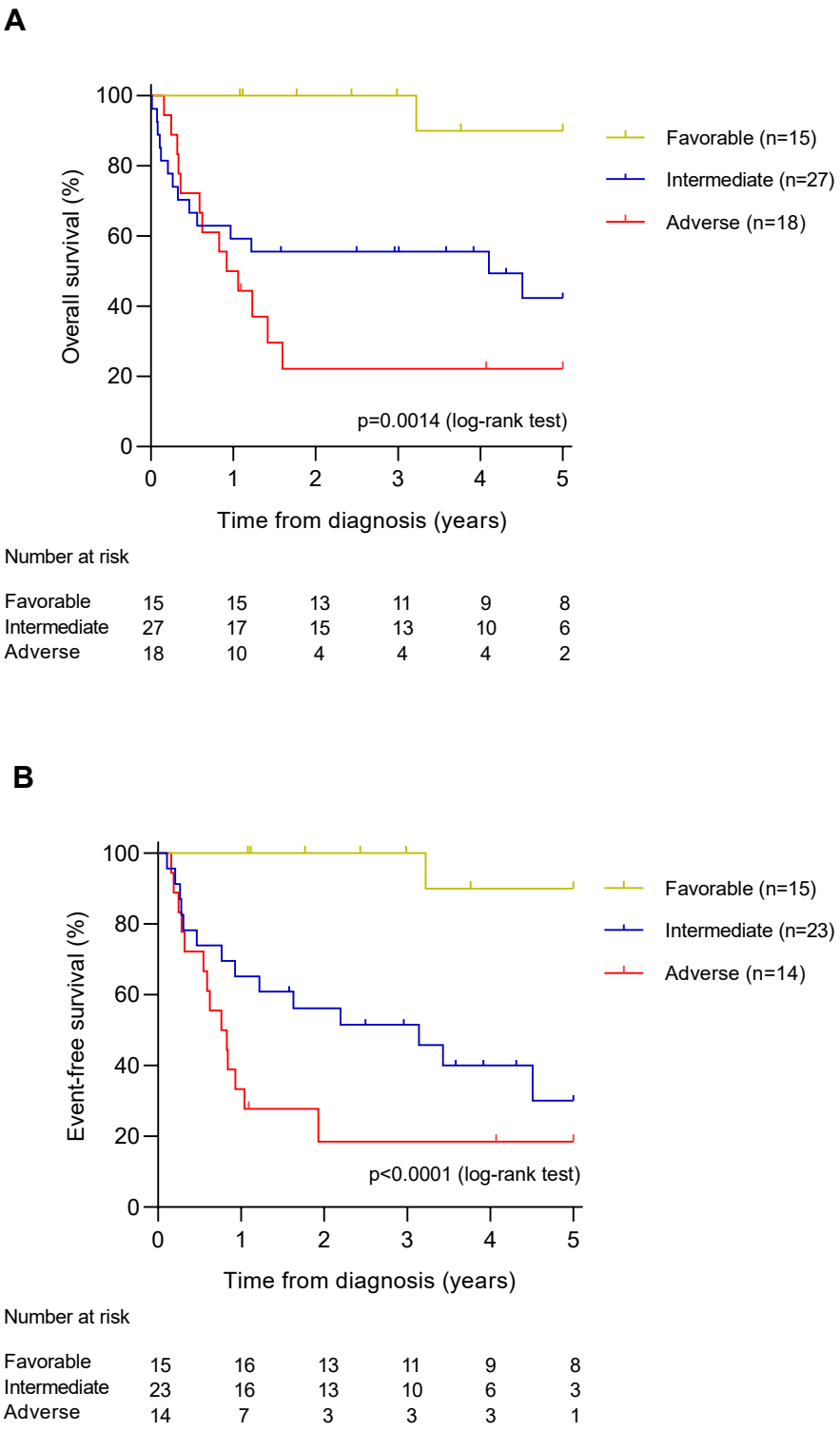


**Figure 16.** Five-year (a) event-free survival and (b) overall survival rates according to favorable (yellow), intermediate (blue), and adverse (red) risk groups based on cytogenetic alterations and mutational status *of NPM1*, *CEBPA*, *FLT3*-ITD, and *WT1*. Log-rank P value is indicated.

In our cohort, 25.3% (19/75) of patients experienced relapse. Our cohort is representative for patients with relapsed AML in terms of time to relapse (TTR) rates, as approximately half of the relapses (57.9%; 11/19) were early relapses, occurring within 1 year after diagnosis. Comparing the 3-year OS of patients with early and late relapse from the time of relapse revealed significantly better prognosis for patients with late relapse (87.5% vs. 9.1%; p=0.0004; log-rank test) (Figure 17).

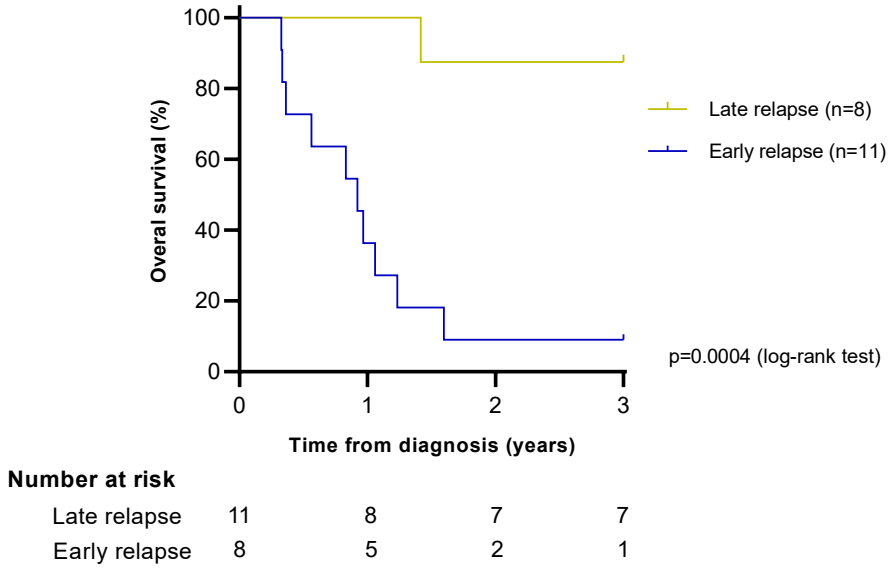


**Figure 17.** Three-year overall survival rates of patients with early (blue) and late (yellow) relapses. OS was calculated from the time of relapse. Patients with late relapses had significantly superior survival. Log-rank P value is indicated.

### IV.2. Assessment of acquired mutations in inherited myeloid malignancies

Targeted NGS with a mean allelic depth of 1,300 revealed in total 78 acquired mutations corresponding to 27 genes. At least one acquired mutation was identified in 28/51 individuals. The median variant allele frequency of the acquired mutations was 11.7% (range, 5.2–99.5%).

To better understand the spectrum of acquired mutations in inherited myeloid disorders, in addition to our cohort, we performed an integrative analysis of the acquired mutations in published families harboring germline variants in the same 13 predisposing loci. Overall, 395 MDS/AML patients from 64 publications were included in the analysis. The integrated results showed marked heterogeneity of acquired mutations in familial myeloid malignancies. We found a high frequency of second-hit *RUNX1*, *CEBPA*, and *DDX41* mutations in patients harboring these germline mutations. Monosomy 7, trisomy 8, *STAG2* and *ASXL1* mutations were recurrently detected in *GATA2* families, while *CEBPA* families usually acquired secondary *GATA2* mutations. Acquired *TP53* mutations were present in a strikingly high 75% of patients with germline *SBDS* mutation, several of whom encountered multiple *TP53* mutations.

Our study reveals a distinctive mutational pattern in *TERT* families for the first time. Four families (FML) with germline *TERT* variants were included in our series (FML038, FML039, FML040 and FML041), with overall six patients with aplastic anemia (AA) (n=2), MDS (n=3) and AML (n=1), and three asymptomatic carriers.

Figure 18 shows a schematic representation of the location of the four *TERT* germline variants: FML038 (p.Arg83Pro) and FML041 (p.His482Profs*27) harbor heterozygous missense and truncating variants, respectively, within the RNA interacting domains, while families FML039 (p.Arg631Gln) and FML040 (p.Pro785Leu) retained heterozygous missense *TERT* variants located within catalytic reverse transcriptase domain (RTD) responsible for maintaining telomere ends. A previous study showed that carriers of the RTD *TERT* variants in FML039 and FML040 exhibit short telomeres and abolished/reduced telomerase activity (161).

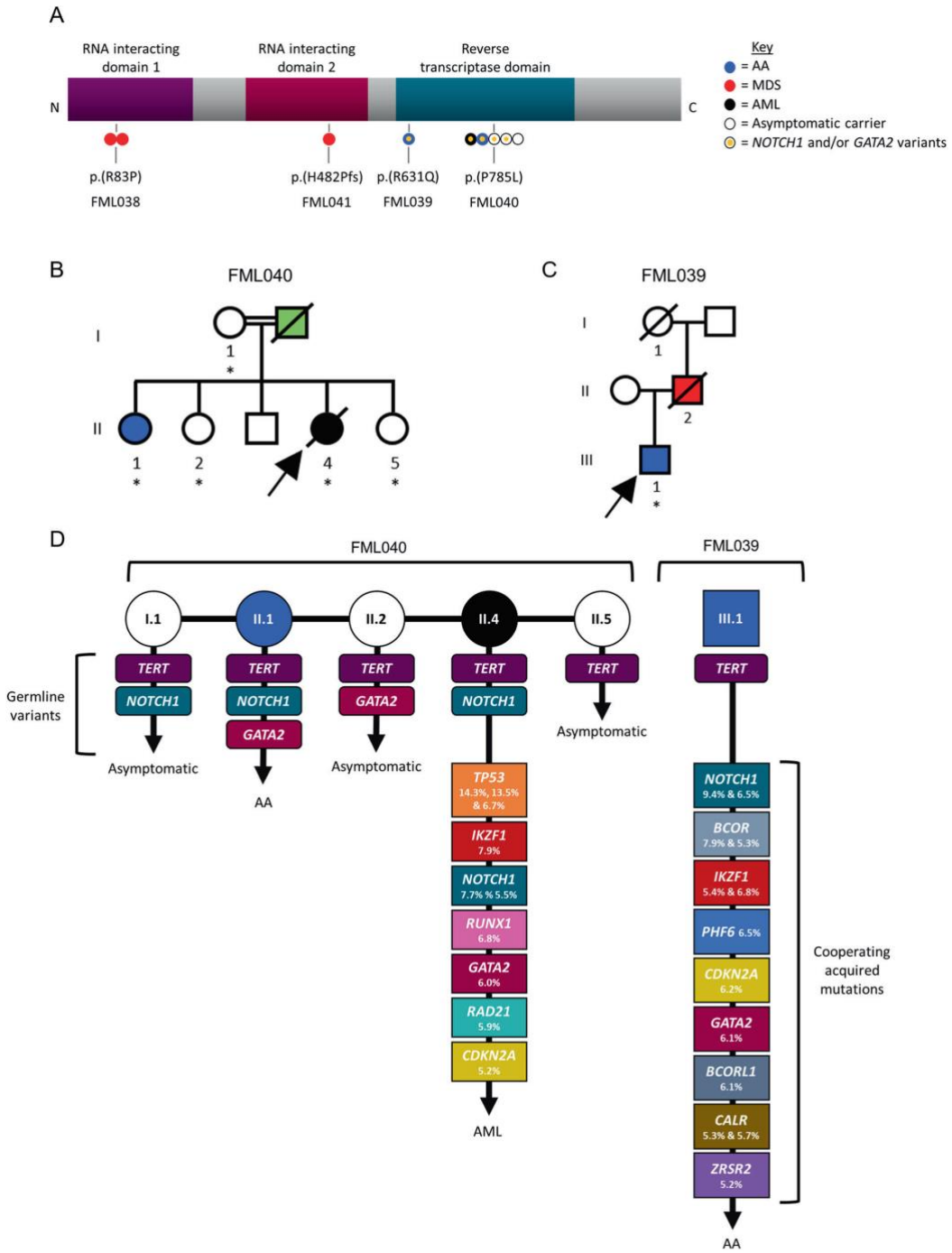


**Figure 18.** Analysis of *TERT* families. (a) Schematic of the *TERT* protein. The location of germline variants of 9 individuals in our series is depicted in relation to the RNA-interacting and catalytic domain(s). Circles represent individual cases and are color-coded by disease phenotype. (b)(c) Schematic representation of *TERT* families, FML040 and FML039. Black represents AML; red, MDS; blue, bone marrow failure; and green, other cancers. Samples included for targeted sequencing are indicated with an asterisk. (d) Variants identified in *TERT* families, FML040 and FML039. Germline variant pattern (*TERT*, *GATA2*, *NOTCH1*), cooperating acquired mutations with corresponding VAF and patient phenotype (AML, AA or asymptomatic) are indicated.

A complex pattern of inheritance was observed in FML040, suggesting that germline variants at three discrete loci, including *TERT*, *NOTCH1*, and *GATA2*, may determine the clinical presentation in this family (Figure 18D). Two offspring inherited *TERT* and *NOTCH1* (p.Pro2128Leu) variants from the asymptomatic mother, including the index case who developed secondary AML (II.4), and her sister diagnosed with AA (II.1). Sibling II.1 also inherited a germline *GATA2* variant (p.Pro14Ser) that was also present in her asymptomatic sister (II.2) which we assume was inherited from her father (I.II). Interestingly, in the index case with secondary AML (II.4) acquired mutations in *NOTCH1* (p.Glu1636Lys, Ala1634Asp) and *GATA2* (p.Phe400Leu) at low VAF could also be detected. Therefore, our current model proposes that the onset of overt symptoms may depend on the presence of both *NOTCH1* and *GATA2* variants since the symptom-free mother (I.1) harbors a single *NOTCH1* variant and the asymptomatic sibling (II.2) carries a single *GATA2* variant. In support of this model, we found that the index case in family FML039 (III.1), with AA and having a germline *TERT* variant, also retained two acquired *NOTCH1* mutations (p.Pro2551fs, p.Ser2499delinsSerPro) and a *GATA2* mutation (p.Glu398Lys) with low VAFs.

## V. DISCUSSION

Although the treatment efficacy of AML has improved during the past decades, primarily owing to increasingly intensified regimens, refined allograft indications, and supportive care, the clinical outcomes have plateaued, with 70% of patients achieving 5-year survival (134, 162, 163). The genomic background of AML has been widely studied; however, pediatric AML is less characterized as only a limited number of studies focused on investigating the molecular landscape of children with AML at diagnosis (101, 119, 164-168). Genomic profiling of pediatric AML unveiled a low tumor mutational burden similar to adult patients, with some shared recurrently mutated genes. Nevertheless, the spectrum of genetic alterations in pediatric and adult AML patients are remarkably different, suggesting that the pathogenesis of pediatric AML differs from that of adult AML (115, 164). Consequently, not all findings related to adult AML patients apply to children, justifying the need to further investigate the mutation profiles of children with AML.

Clonal chromosomal abnormalities are more common in pediatric AML compared to adult cases, and many of these cytogenetic alterations have important implications for prognosis (7, 58, 126, 169). The results of this study indicate that the distribution of patients across different cytogenetic categories was in line with previous reports as abnormal karyotype was detected in 77.5% (55/71) of pediatric AML patients, and *KMT2A*-rearrangement and normal karyotype were the two most observed genotypes. Due to the limited number of patients, analyzing the prognostic implications of individual chromosomal abnormalities was not feasible. However, using cytogenetics for risk classification effectively differentiated between groups with favorable, intermediate, and adverse risks in our cohort.

Through the panel-based targeted NGS approach, we detected SNVs/indels in 83.8% of patients. Furthermore, when these results were combined with cytogenetic data, we observed at least one molecular aberration in 98.6% of patients.

Although the study focused on only a limited number of genes, the distinct combination of mutations resulted in a unique genomic profile for each patient. As expected, the

number of variants per case was relatively low, reflecting the low tumor mutational burden previously observed in pediatric AML(164, 165).

Patients with *KMT2A*-rearrangement had fewer mutations than other subtypes of AML in accordance with previously published studies (101, 167). *KMT2A*-rearrangement is associated with early onset AML; however, in our cohort, more than half of patients with *KMT2A*-rearrangement were older than 2 years. An increased number of mutations was detected in patients with normal karyotypes and karyotypes associated with adverse prognosis. In our cohort, the most common class of mutations involved genes that control kinase signaling and encode transcription factors, while mutations in epigenetic components or spliceosome complexes commonly occurring in adult AML patients were present infrequently, in line with the literature (21, 170, 171). Comparison of the mutation landscape revealed significant differences in the mutational frequencies in some affected genes (Figure 19.).

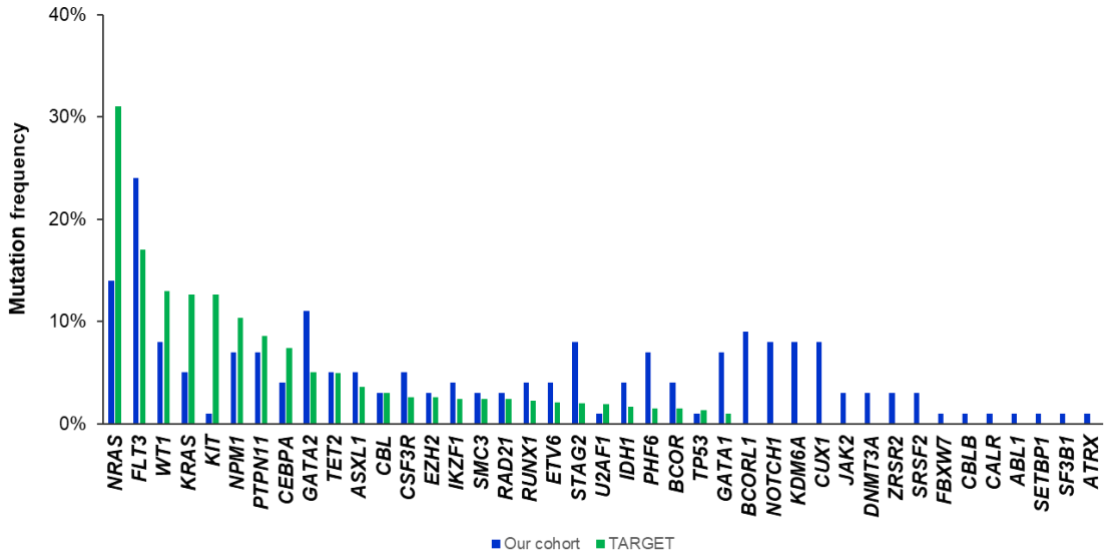


**Figure 19.** Differences in mutation frequencies of key myeloid genes in our cohort compared with TARGET (Therapeutically Applicable Research to Generate Effective Treatments) AML results. It should be noted that some of the differences in individual gene mutation frequencies between the two cohorts may be attributed to the different sequencing technologies (deep targeted next-generation sequencing versus whole-exome sequencing) used in these studies, as well as to the limited size (n = 75) of our patient cohort. Notably, our cohort included five cases of Down syndrome acute megakaryocytic leukemia and three cases of APL, whereas these entities were absent from the TARGET AML cohort.

RAS pathway mutations (*NRAS*, *KRAS*, *PTPN11*, *CBL)* occurred less commonly in our cohort as 27% of patients carried a RAS pathway mutation at diagnosis compared to 40-50% frequencies reported in the previous publications (101, 115, 164, 172). *KIT* mutations were less frequently observed with only a single CBF-AML patient carrying *KIT* mutation, while *KIT* mutations are detected at a significantly higher ratio (20-40%) in pediatric CBF-AML patients in the literature (67, 68, 173).

In our cohort, *BCORL1, CUX1, KDM6A, PHF6,* and *STAG2* mutations were detected at a higher frequency than in any other previous publications predominantly using whole-genome sequencing (WGS) and whole-exome sequencing (WES) (101, 115, 164, 174). Mutations in *BCOR* and its homologue, *BCORL1* were first described in adult AML presenting in 5-10% of patients - mostly detected in secondary- and therapy-related AML - associated with inferior outcomes (175-177), while in children with AML, *BCOR* / *BCORL1* mutations were present in only 2.9-3.4% of patients (101, 164, 167, 174). Assessment of clonality using VAF values suggested that the vast majority of *BCOR*/*BCORL1* mutations were subclonal that might explain the significantly higher frequency of *BCOR*/*BCORL1* mutations in our cohort.

Interestingly, *CUX1* mutations detected in 8% of our cohort were not identified in the previous WGS/WES-based pediatric AML studies and were detected only in a single patient with pediatric AML by Tarlock et al. using deep-sequencing (115, 119, 164, 167). *CUX1* is a transcription factor regulating cell cycle progression and apoptosis and acts as a haplo-insufficient tumor suppressor frequently impaired in myeloid neoplasms, mainly through loss of chromosome 7 (21, 178, 179). *CUX1* mutations are associated with inferior prognosis in myeloid malignancies, similar to the adverse outcome of -7/(del7q) myeloid malignancies (178, 180). The higher frequency of *CUX1* mutations detected in our cohort may be explained by the usage of deep sequencing, which enabled us to identify *CUX1* mutations even at a subclonal level.

*KDM6A* was recurrently altered in our cohort (8.1%) and associated with *RUNX1::RUNX1T1*-rearrangement, in contrast with previous publications where *KDM6A* mutations were only described in 0.5-3% of pediatric AML patients and

enrichment of *KDM6A* mutation has not been previously described in children with CBF-AML (115, 119, 168). Nevertheless, studies on adult CBF-AML showed that mutations of chromatin modifiers (including *KDM6A* as well) were observed almost exclusively in AML with *RUNX1::RUNX1T1* fusion (65, 181).

Another X-linked tumor suppressor gene recurrently mutated in our cohort was *PHF6*. *PHF6* mutations are rare events in pediatric AML as only 1.4-3.6% of patients harbor *PHF6* mutations with association with poor outcomes (101, 164, 182). Recently, Stratmann et al. demonstrated that the mutational frequency of *PHF6* was substantially higher in relapsed or primary resistant patients than previously reported in diagnosis-only cohorts (116). This finding supports the association of *PHF6* mutations with poor outcomes, also reported in our study and by Marceau-Renaut et al. (101).

Risk stratification of pediatric AML is mostly based on cytogenetics; however, some recurrent mutations such as *NPM1*, *CEBPA, FLT3-ITD* with *WT1* are also included in most risk classifications (4, 183). Recently, several publications suggested the optimization of risk stratifications of AML by incorporating additional genetic lesions (184-187). The newly published risk classification of the European LeukemiaNet recommends mutational analysis of genes including *BCOR*, *EZH2*, *SF3B1*, *SRSF2*, *STAG2*, *U2AF1* and *ZRSR2* as these alterations are associated with high-risk features and adverse prognosis (104). Unfortunately, prognostic stratifications developed for adults are not necessarily suitable for children. Nonetheless, these initiatives draw attention to the clinical need to further investigate how newly identified molecular alterations of pediatric AML could be incorporated in the current risk classifications. Most studies in the field of pediatric AML focused on the prognostic significance of a single gene or a subset of genes; however, with these approaches, the true heterogeneity of pediatric AML could not be captured. Only a limited number of studies evaluated the prognostic significance of mutations in childhood AML in the era of high-throughput sequencing. The favorable prognostic impact of *NPM1* and *CEBPA* mutations has been well established for many years, while the prognostic significance of other molecular alterations is less defined (112, 127, 188, 189). Marceau-Renaut et al. demonstrated that *PHF6* and *RUNX1* mutations are associated with poor prognosis in childhood AML,

while Umeda et al. showed that *UBTF* tandem duplication is a recurrent lesion in pediatric AML and associated with poor outcomes (101, 117). Due to the limited size of our cohort, the prognostic significance of individual mutations could not be comprehensively investigated; still, results from our current analysis suggest that tumor suppressor gene mutations (*PHF6*, *TP53*, *WT1*) are associated with induction failure and a trend toward shorter EFS, although these findings will need to be confirmed in independent, larger studies.

Our study also compared the mutational landscape of 8 matched diagnosis and relapse pediatric AML samples using panel-based targeted NGS and revealed a slightly higher number of mutations at relapse compared to the diagnostic samples and mutations persisting at relapse had higher VAF at diagnosis compared with those that were eliminated. Relapse-specific mutation could not be identified. However, mutations in tumor suppressor genes and epigenetic modifiers occurred more frequently at relapse. In five patients, relapsed AML evolved from one of the subclones detected at the initial diagnosis and was accompanied by several additional mutations that were absent or present at a lower allele frequency in the diagnostic sample, indicating the multistep process of leukemia recurrence. In contrast, two patients relapsed early with seemingly identical clones suggesting the incomplete eradication of leukemic cells by initial treatment. Comparing the clinical outcomes of patients with early and late relapse unveiled that patients with early relapse have significantly worse prognosis, suggesting that identification and quantification of MRD may be of great importance as early intervention and change of therapy may improve the clinical outcome of these patients.

Inherited myeloid malignancy is considered a rare disease entity that exhibits significant heterogeneity in penetrance, age of onset, and clinical presentation. Genetic analysis of affected families has clearly demonstrated that causative germline variants represent just one piece of the puzzle, and to understand the clinical heterogeneity seen even within families, it is inevitable to uncover the landscape of acquired mutations. In an international collaboration, we studied the somatic mutational landscape of familial myeloid malignancies, integrating the somatic profiling of 446 individuals from this study with previously published families with inherited variants in 13 predisposition gene loci. Our analysis demonstrated significant heterogeneity in acquired mutations and confirmed

previous observations of the striking frequency of second-hit *RUNX1*, *CEBPA*, and *DDX41* mutations in patients harboring these germline variants (158). While we observed enrichment of distinct acquired genetic alterations in specific subgroups, such as recurrence of *STAG2* and *ASXL1* in *GATA2* families, *GATA2* mutations in *CEBPA* families, and *TP53* mutations in patients with *SBDS* mutation, because of the heterogeneity of acquired mutations, their overall frequency was low. Therefore, recognizing the relevance of these infrequent secondary variants in this relatively rare entity is challenging.

Analysis of four families with germline *TERT* variants revealed that the combination of *NOTCH1* and *GATA2* may act as a risk factor for development of myeloid disease. Although the exact molecular mechanism for the functional interaction between *TERT*, *NOTCH1* and *GATA2* has not been elucidated, it has previously been demonstrated that telomerase affects *NOTCH1* signaling (190) while *NOTCH1* directly controls the expression of *GATA2* (191). Further studies are needed to assess whether acquired *NOTCH1* and *GATA2* mutations play a crucial role in disease progression or if other related mutations can also be found in families with germline *TERT* variants.

Our dataset has provided insights into the complex interplay between genetic lesions that may contribute to disease development, particularly in the case of *TERT*-mutated families, suggesting that a particular combination of germline and somatic co-occurring variants may shape disease progression. Above all, there is an unmet need to include familial loci for analysis in routine diagnostic panels to gain a better understanding of the pathogenesis of both familial and sporadic disease.

## VI. CONCLUSIONS

The conclusions of my work are the following:

- We determined the mutational profile of a relatively large cohort of Hungarian children diagnosed with AML by targeted next-generation sequencing for the first time.
- Three novel mutations in *NPM1* and *CEBPA* genes and a recurrent *BCORL1* mutation in three patients were identified.
- *BCORL1*, *CUX1*, *KDM6A*, *PHF6*, and *STAG2* were found to be more frequently mutated in pediatric AML than previously appreciated. Low VAFs of these mutations suggest that these alterations are late events in leukemogenesis.
- *CUX1* was recurrently mutated in cytogenetically normal AML, while *KDM6A* mutations are significantly enriched in AML with *RUNX1::RUNX1T1* fusion.
- Mutations of tumor suppressor genes (*PHF6*, *TP53*, *WT1*) were found to be associated with induction failure and a trend towards shorter EFS.
- Comparing samples from diagnosis and relapse revealed significant enrichment of mutations in tumor suppressor genes and genes involved in epigenetic modification at relapse.
- Acquired mutational landscape of individuals with germline predisposition to myeloid malignancy showed marked heterogeneity.
- Our study identified a novel mutational signature in *TERT* families for the first time.
- The combination of *NOTCH1* and *GATA2* mutations may act as a risk factor for developing myeloid disease in patients with germline *TERT* variants.

## VII. SUMMARY

Our study focused on molecular genetic profiling of pediatric acute myeloid leukemia (AML) and inherited myeloid malignancies using a panel-based targeted next-generation sequencing (NGS) approach.

We performed an integrative analysis of cytogenetic and molecular profiles of pediatric AML from a multicentric, real-world patient cohort. To the best of our knowledge, our study is the first to characterize the mutational landscape of pediatric AML patients treated according to AML BFM protocols in Central-Eastern Europe. Our results demonstrate that childhood AML represents a distinct entity that differs from adult AML regarding the spectrum of gene mutations. We identified three novel mutations in *NPM1* and *CEBPA* genes and a recurrent *BCORL1* mutation in three patients. We demonstrated that *BCORL1*, *CUX1*, *KDM6A*, *PHF6*, and *STAG2* genes were more frequently mutated in pediatric AML than previously appreciated in earlier studies. Mutations of tumor suppressor genes were associated with induction failure and a trend towards shorter EFS; moreover, they were enriched in relapse samples alongside mutations of genes involved in epigenetic modification. Our results suggest that targeted NGS is a robust tool for the reliable detection of disease-relevant alterations that determine distinct genetic subgroups of pediatric AML and are also associated with disease prognosis.

The somatic mutational landscapes of 51 individuals from 33 families with inherited myeloid malignancy were analyzed using a panel-based targeted NGS approach. Our analysis demonstrated significant heterogeneity in acquired mutations that explains the clinical heterogeneity observed even within families with the same germline variant. Our dataset has provided insights into the complex interplay between genetic lesions that may contribute to disease development. We identified a novel mutational signature in *TERT* families for the first time. The combination of *NOTCH1* and *GATA2* mutations may act as a risk factor for developing myeloid disease in patients with germline *TERT* variants. Overall, our results demonstrate that a particular combination of germline and somatic co-occurring variants may shape disease progression. Therefore, the investigation of acquired mutations in inherited myeloid malignancies is essential to understand the reason behind the significant heterogeneity in penetrance, age of onset, and clinical outcome.

## VIII. REFERENCES


1. Siegel RL, Giaquinto AN, Jemal A. Cancer statistics, 2024. CA Cancer J Clin. 2024;74(1):12-49.

2. SEER*Explorer: An interactive website for SEER cancer statistics [Internet]. Surveillance Research Program, National Cancer Institute; 2023 Apr 19. [updated: 2023 Jun 8; cited 2023 Oct 19]. Available from: https://seer.cancer.gov/statistics-network/explorer/. Data source(s): SEER Incidence Data, November 2022 Submission (1975-2020), SEER 22 registries. [

3. Siegel DA, King JB, Lupo PJ, Durbin EB, Tai E, Mills K, et al. Counts, incidence rates, and trends of pediatric cancer in the United States, 2003-2019. J Natl Cancer Inst. 2023;115(11):1337-54.

4. Creutzig U, van den Heuvel-Eibrink MM, Gibson B, Dworzak MN, Adachi S, de Bont E, et al. Diagnosis and management of acute myeloid leukemia in children and adolescents: recommendations from an international expert panel. Blood. 2012;120(16):3187-205.

5. Garami M, Schuler D, Jakab Z. [Importance of the National Childhood Cancer Registry in the field of paediatric oncology care in Hungary]. Orv Hetil. 2014;155(19):732-9.

6. Miller KD, Nogueira L, Devasia T, Mariotto AB, Yabroff KR, Jemal A, et al. Cancer treatment and survivorship statistics, 2022. CA Cancer J Clin. 2022;72(5):409-36.

7. Quessada J, Cuccuini W, Saultier P, Loosveld M, Harrison CJ, Lafage-Pochitaloff M. Cytogenetics of Pediatric Acute Myeloid Leukemia: A Review of the Current Knowledge. Genes (Basel). 2021;12(6).

8. Howlader N NA, Krapcho M, Miller D, Brest A, Yu M, Ruhl J, Tatalovich Z, Mariotto A, Lewis DR, Chen HS, Feuer EJ, Cronin KA (eds). SEER Cancer Statistics Review, 1975-2017, National Cancer Institute. Bethesda, MD, https://seer.cancer.gov/csr/1975_2017/, based on November 2019 SEER data submission, posted to the SEER web site, April 2020. [

9. Jani CT, Ahmed A, Singh H, Mouchati C, Al Omari O, Bhatt PS, et al. Burden of AML, 1990-2019: Estimates From the Global Burden of Disease Study. JCO Glob Oncol. 2023;9:e2300229.

10. Dong Y, Shi O, Zeng Q, Lu X, Wang W, Li Y, et al. Leukemia incidence trends at the global, regional, and national level between 1990 and 2017. Exp Hematol Oncol. 2020;9:14.

11. Chen X, Pan J, Wang S, Hong S, Hong S, He S. The Epidemiological Trend of Acute Myeloid Leukemia in Childhood: a Population-Based Analysis. J Cancer. 2019;10(20):4824-35.

12. Advani PG, Schonfeld SJ, Curtis RE, Dores GM, Linet MS, Sigel BS, et al. Risk of therapy-related myelodysplastic syndrome/acute myeloid leukemia after childhood cancer: a population-based study. Leukemia. 2019;33(12):2947-78.

13. Brown CA, Youlden DR, Aitken JF, Moore AS. Therapy-related acute myeloid leukemia following treatment for cancer in childhood: A population-based registry study. Pediatr Blood Cancer. 2018;65(12):e27410.

14. Tomizawa D, Tsujimoto SI. Risk-Stratified Therapy for Pediatric Acute Myeloid Leukemia. Cancers. 2023;15(16).

15. Rasche M, Zimmermann M, Borschel L, Bourquin JP, Dworzak M, Klingebiel T, et al. Successes and challenges in the treatment of pediatric acute myeloid leukemia: a retrospective analysis of the AML-BFM trials from 1987 to 2012. Leukemia. 2018;32(10):2167-77.

16. Rubnitz JE, Inaba H, Dahl G, Ribeiro RC, Bowman WP, Taub J, et al. Minimal residual disease-directed therapy for childhood acute myeloid leukaemia: results of the AML02 multicentre trial. Lancet Oncol. 2010;11(6):543-52.

17. Abrahamsson J, Forestier E, Heldrup J, Jahnukainen K, Jonsson OG, Lausen B, et al. Response-guided induction therapy in pediatric acute myeloid leukemia with excellent remission rate. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2011;29(3):310-5.

18. Gibson BE, Webb DK, Howman AJ, De Graaf SS, Harrison CJ, Wheatley K, et al. Results of a randomized trial in children with Acute Myeloid Leukaemia: medical research council AML12 trial. British journal of haematology. 2011;155(3):366-76.

19. Bonaventure A, Harewood R, Stiller CA, Gatta G, Clavel J, Stefan DC, et al. Worldwide comparison of survival from childhood leukaemia for 1995-2009, by subtype, age, and sex (CONCORD-2): a population-based study of individual data for 89 828 children from 198 registries in 53 countries. Lancet Haematol. 2017;4(5):e202-e17.

20. Pui CH, Campana D, Pei D, Bowman WP, Sandlund JT, Kaste SC, et al. Treating childhood acute lymphoblastic leukemia without cranial irradiation. The New England journal of medicine. 2009;360(26):2730-41.
21. Papaemmanuil E, Gerstung M, Bullinger L, Gaidzik VI, Paschka P, Roberts ND, et al. Genomic Classification and Prognosis in Acute Myeloid Leukemia. The New England journal of medicine. 2016;374(23):2209-21.
22. Patel JP, Gönen M, Figueroa ME, Fernandez H, Sun Z, Racevskis J, et al. Prognostic relevance of integrated genetic profiling in acute myeloid leukemia. The New England journal of medicine. 2012;366(12):1079-89.
23. Miles LA, Bowman RL, Merlinsky TR, Csete IS, Ooi AT, Durruthy-Durruthy R, et al. Single-cell mutation analysis of clonal evolution in myeloid malignancies. Nature. 2020;587(7834):477-82.
24. Teepen JC, Curtis RE, Dores GM, Berrington de Gonzalez A, van den Heuvel-Eibrink MM, Kremer LCM, et al. Risk of subsequent myeloid neoplasms after radiotherapy treatment for a solid cancer among adults in the United States, 2000-2014. Leukemia. 2018;32(12):2580-9.
25. Shallis RM, Wang R, Davidoff A, Ma X, Zeidan AM. Epidemiology of acute myeloid leukemia: Recent progress and enduring challenges. Blood Rev. 2019;36:70-87.
26. Boddu P, Kantarjian HM, Garcia-Manero G, Ravandi F, Verstovsek S, Jabbour E, et al. Treated secondary acute myeloid leukemia: a distinct high-risk subset of AML with adverse prognosis. Blood advances. 2017;1(17):1312-23.
27. Granfeldt Ostgard LS, Medeiros BC, Sengelov H, Norgaard M, Andersen MK, Dufva IH, et al. Epidemiology and Clinical Significance of Secondary and Therapy-Related Acute Myeloid Leukemia: A National Population-Based Cohort Study. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2015;33(31):3641-9.
28. Hulegardh E, Nilsson C, Lazarevic V, Garelius H, Antunovic P, Rangert Derolf A, et al. Characterization and prognostic features of secondary acute myeloid leukemia in a population-based setting: a report from the Swedish Acute Leukemia Registry. Am J Hematol. 2015;90(3):208-14.

29. Houot J, Marquant F, Goujon S, Faure L, Honore C, Roth MH, et al. Residential Proximity to Heavy-Traffic Roads, Benzene Exposure, and Childhood Leukemia-The GEOCAP Study, 2002-2007. Am J Epidemiol. 2015;182(8):685-93.
30. Belson M, Kingsley B, Holmes A. Risk factors for acute leukemia in children: a review. Environ Health Perspect. 2007;115(1):138-45.
31. Yang F, Long N, Anekpuritanang T, Bottomly D, Savage JC, Lee T, et al. Identification and prioritization of myeloid malignancy germline variants in a large cohort of adult patients with AML. Blood. 2022;139(8):1208-21.
32. Guidugli L, Johnson AK, Alkorta-Aranburu G, Nelakuditi V, Arndt K, Churpek JE, et al. Clinical utility of gene panel-based testing for hereditary myelodysplastic syndrome/acute leukemia predisposition syndromes. Leukemia. 2017;31(5):1226-9.
33. Morton LM, Dores GM, Schonfeld SJ, Linet MS, Sigel BS, Lam CJK, et al. Association of Chemotherapy for Solid Tumors With Development of Therapy-Related Myelodysplastic Syndrome or Acute Myeloid Leukemia in the Modern Era. Jama Oncology. 2019;5(3):318-25.
34. Mauritzson N, Albin M, Rylander L, Billstrom R, Ahlgren T, Mikoczy Z, et al. Pooled analysis of clinical and cytogenetic features in treatment-related and de novo adult acute myeloid leukemia and myelodysplastic syndromes based on a consecutive series of 761 patients analyzed 1976-1993 and on 5098 unselected cases reported in the literature 1974-2001. Leukemia. 2002;16(12):2366-78.
35. Seedhouse C, Russell N. Advances in the understanding of susceptibility to treatment-related acute myeloid leukaemia. British journal of haematology. 2007;137(6):513-29.
36. Kayser S, Dohner K, Krauter J, Kohne CH, Horst HA, Held G, et al. The impact of therapy-related acute myeloid leukemia (AML) on outcome in 2853 adult patients with newly diagnosed AML. Blood. 2011;117(7):2137-45.
37. Nilsson C, Linde F, Hulegardh E, Garelius H, Lazarevic V, Antunovic P, et al. Characterization of therapy-related acute myeloid leukemia: increasing incidence and prognostic implications. Haematologica. 2023;108(4):1015-25.
38. McNerney ME, Godley LA, Le Beau MM. Therapy-related myeloid neoplasms: when genetics and environment collide. Nat Rev Cancer. 2017;17(9):513-27.

39. Churpek JE, Marquez R, Neistadt B, Claussen K, Lee MK, Churpek MM, et al. Inherited mutations in cancer susceptibility genes are common among survivors of breast cancer who develop therapy-related leukemia. Cancer. 2016;122(2):304-11.
40. Schulz E, Valentin A, Ulz P, Beham-Schmid C, Lind K, Rupp V, et al. Germline mutations in the DNA damage response genes BRCA1, BRCA2, BARD1 and TP53 in patients with therapy related myeloid neoplasms. J Med Genet. 2012;49(7):422-8.
41. Kim B, Yun W, Lee ST, Choi JR, Yoo KH, Koo HH, et al. Prevalence and clinical implications of germline predisposition gene mutations in patients with acute myeloid leukemia. Sci Rep. 2020;10(1):14297.
42. Maese LD, Wlodarski MW, Kim SY, Bertuch AA, Bougeard G, Chang VY, et al. Update on Recommendations for Surveillance for Children with Predisposition to Hematopoietic Malignancy. Clinical cancer research : an official journal of the American Association for Cancer Research. 2024;30(19):4286-95.
43. Samaraweera SE, Wang PPS, Li KL, Casolari DA, Feng J, Pinese M, et al. Childhood acute myeloid leukemia shows a high level of germline predisposition. Blood. 2021;138(22):2293-8.
44. Jeong D, Lee DS, Kim N, Choi S, Kim K, Kim SM, et al. Prevalence of germline predisposition gene mutations in pediatric acute myeloid leukemia: Genetic background of pediatric AML. Leuk Res. 2019;85:106210.
45. Kahn JD, Miller PG, Silver AJ, Sellar RS, Bhatt S, Gibson C, et al. PPM1D-truncating mutations confer resistance to chemotherapy and sensitivity to PPM1D inhibition in hematopoietic cells. Blood. 2018;132(11):1095-105.
46. Boettcher S, Miller PG, Sharma R, McConkey M, Leventhal M, Krivtsov AV, et al. A dominant-negative effect drives selection of TP53 missense mutations in myeloid malignancies. Science. 2019;365(6453):599-604.
47. Wong TN, Ramsingh G, Young AL, Miller CA, Touma W, Welch JS, et al. Role of TP53 mutations in the origin and evolution of therapy-related acute myeloid leukaemia. Nature. 2015;518(7540):552-5.
48. Takahashi K, Wang F, Kantarjian H, Doss D, Khanna K, Thompson E, et al. Preleukaemic clonal haemopoiesis and risk of therapy-related myeloid neoplasms: a case-control study. Lancet Oncol. 2017;18(1):100-11.

49. Schwartz JR, Ma J, Kamens J, Westover T, Walsh MP, Brady SW, et al. The acquisition of molecular drivers in pediatric therapy-related myeloid neoplasms. Nat Commun. 2021;12(1):985.
50. Eaves CJ. Hematopoietic stem cells: concepts, definitions, and the new reality. Blood. 2015;125(17):2605-13.
51. Döhner H, Weisdorf DJ, Bloomfield CD. Acute Myeloid Leukemia. The New England journal of medicine. 2015;373(12):1136-52.
52. Kelly LM, Gilliland DG. Genetics of myeloid leukemias. Annu Rev Genomics Hum Genet. 2002;3:179-98.
53. Ley TJ, Miller C, Ding L, Raphael BJ, Mungall AJ, Robertson A, et al. Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. The New England journal of medicine. 2013;368(22):2059-74.
54. Welch JS. Mutation position within evolutionary subclonal architecture in AML. Semin Hematol. 2014;51(4):273-81.
55. Morita K, Wang F, Jahn K, Hu T, Tanaka T, Sasaki Y, et al. Clonal evolution of acute myeloid leukemia revealed by high-throughput single-cell genomics. Nat Commun. 2020;11(1):5327.
56. Ediriwickrema A, Aleshin A, Reiter JG, Corces MR, Kohnke T, Stafford M, et al. Single-cell mutational profiling enhances the clinical evaluation of AML MRD. Blood advances. 2020;4(5):943-52.
57. Tarlock K, Meshinchi S. Pediatric acute myeloid leukemia: biology and therapeutic implications of genomic variants. Pediatr Clin North Am. 2015;62(1):75-93.
58. Creutzig U, Zimmermann M, Reinhardt D, Rasche M, von Neuhoff C, Alpermann T, et al. Changes in cytogenetics and molecular genetics in acute myeloid leukemia from childhood to adult age groups. Cancer. 2016;122(24):3821-30.
59. Speck NA, Gilliland DG. Core-binding factors in haematopoiesis and leukaemia. Nat Rev Cancer. 2002;2(7):502-13.
60. Paschka P, Du J, Schlenk RF, Gaidzik VI, Bullinger L, Corbacioglu A, et al. Secondary genetic lesions in acute myeloid leukemia with inv(16) or t(16;16): a study of the German-Austrian AML Study Group (AMLSG). Blood. 2013;121(1):170-7.

61. Jahn N, Terzer T, Strang E, Dolnik A, Cocciardi S, Panina E, et al. Genomic heterogeneity in core-binding factor acute myeloid leukemia and its clinical implication. Blood advances. 2020;4(24):6342-52.

62. Klein K, Kaspers G, Harrison CJ, Beverloo HB, Reedijk A, Bongers M, et al. Clinical Impact of Additional Cytogenetic Aberrations, cKIT and RAS Mutations, and Treatment Elements in Pediatric t(8;21)-AML: Results From an International Retrospective Study by the International Berlin-Frankfurt-Munster Study Group. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2015;33(36):4247-58.

63. von Neuhoff C, Reinhardt D, Sander A, Zimmermann M, Bradtke J, Betts DR, et al. Prognostic impact of specific chromosomal aberrations in a large group of pediatric patients with acute myeloid leukemia treated uniformly according to trial AML-BFM 98. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2010;28(16):2682-9.

64. Shimada A, Taki T, Tabuchi K, Tawa A, Horibe K, Tsuchida M, et al. KIT mutations, and not FLT3 internal tandem duplication, are strongly associated with a poor prognosis in pediatric acute myeloid leukemia with t(8;21): a study of the Japanese Childhood AML Cooperative Study Group. Blood. 2006;107(5):1806-9.

65. Duployez N, Marceau-Renaut A, Boissel N, Petit A, Bucci M, Geffroy S, et al. Comprehensive mutational profiling of core binding factor acute myeloid leukemia. Blood. 2016;127(20):2451-9.

66. Faber ZJ, Chen X, Gedman AL, Boggs K, Cheng J, Ma J, et al. The genomic landscape of core-binding factor acute myeloid leukemias. Nat Genet. 2016;48(12):1551-6.

67. Pollard JA, Alonzo TA, Gerbing RB, Ho PA, Zeng R, Ravindranath Y, et al. Prevalence and prognostic significance of KIT mutations in pediatric patients with core binding factor AML enrolled on serial pediatric cooperative trials for de novo AML. Blood. 2010;115(12):2372-9.

68. Goemans BF, Zwaan CM, Miller M, Zimmermann M, Harlow A, Meshinchi S, et al. Mutations in KIT and RAS are frequent events in pediatric core-binding factor acute myeloid leukemia. Leukemia. 2005;19(9):1536-42.

69. Shih LY, Liang DC, Huang CF, Chang YT, Lai CL, Lin TH, et al. Cooperating mutations of receptor tyrosine kinases and Ras genes in childhood core-binding factor acute myeloid leukemia and a comparative analysis on paired diagnosis and relapse samples. Leukemia. 2008;22(2):303-7.

70. Qiu KY, Liao XY, Li Y, Huang K, Xu HG, Fang JP, et al. Outcome and prognostic factors of CBF pediatric AML patients with t(8;21) differ from patients with inv(16). BMC Cancer. 2023;23(1):476.

71. Fan J, Gao L, Chen J, Hu S. Influence of KIT mutations on prognosis of pediatric patients with core-binding factor acute myeloid leukemia: a systematic review and meta-analysis. Transl Pediatr. 2020;9(6):726-33.

72. Kayser S, Kramer M, Martinez-Cuadron D, Grenet J, Metzeler KH, Sustkova Z, et al. Characteristics and outcome of patients with core-binding factor acute myeloid leukemia and FLT3-ITD: results from an international collaborative study. Haematologica. 2022;107(4):836-43.

73. Kaburagi T, Yamato G, Shiba N, Yoshida K, Hara Y, Tabuchi K, et al. Clinical significance of RAS pathway alterations in pediatric acute myeloid leukemia. Haematologica. 2022;107(3):583-92.

74. de The H, Chen Z. Acute promyelocytic leukaemia: novel insights into the mechanisms of cure. Nat Rev Cancer. 2010;10(11):775-83.

75. Kutny MA, Alonzo TA, Abla O, Rajpurkar M, Gerbing RB, Wang YC, et al. Assessment of Arsenic Trioxide and All-trans Retinoic Acid for the Treatment of Pediatric Acute Promyelocytic Leukemia: A Report From the Children's Oncology Group AAML1331 Trial. JAMA Oncol. 2022;8(1):79-87.

76. Taga T, Tomizawa D, Takahashi H, Adachi S. Acute myeloid leukemia in children: Current status and future directions. Pediatr Int. 2016;58(2):71-80.

77. Iland HJ, Collins M, Bradstock K, Supple SG, Catalano A, Hertzberg M, et al. Use of arsenic trioxide in remission induction and consolidation therapy for acute promyelocytic leukaemia in the Australasian Leukaemia and Lymphoma Group (ALLG) APML4 study: a non-randomised phase 2 trial. Lancet Haematol. 2015;2(9):e357-66.

78. Balgobind BV, Raimondi SC, Harbott J, Zimmermann M, Alonzo TA, Auvrignon A, et al. Novel prognostic subgroups in childhood 11q23/MLL-rearranged acute myeloid leukemia: results of an international retrospective study. Blood. 2009;114(12):2489-96.

79. Blais S, Boutroux H, Pasquet M, Leblanc T, Fenneteau O, Gandemer V, et al. Is Acute Myeloblastic Leukemia in Children Under 2 Years of Age a Specific Entity? A Report from the FRENCH ELAM02 Study Group. Hemasphere. 2019;3(6):e316.
80. Meyer C, Hofmann J, Burmeister T, Groger D, Park TS, Emerenciano M, et al. The MLL recombinome of acute leukemias in 2013. Leukemia. 2013;27(11):2165-76.
81. Thorsteinsdottir U, Kroon E, Jerome L, Blasi F, Sauvageau G. Defining roles for HOX and MEIS1 genes in induction of acute myeloid leukemia. Mol Cell Biol. 2001;21(1):224-34.
82. van Weelderen RE, Klein K, Harrison CJ, Jiang Y, Abrahamsson J, Arad-Cohen N, et al. Measurable Residual Disease and Fusion Partner Independently Predict Survival and Relapse Risk in Childhood KMT2A-Rearranged Acute Myeloid Leukemia: A Study by the International Berlin-Frankfurt-Munster Study Group. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2023;41(16):2963-74.
83. van Weelderen RE, Harrison CJ, Klein K, Jiang Y, Abrahamsson J, Alonzo T, et al. Optimized cytogenetic risk-group stratification of KMT2A-rearranged pediatric acute myeloid leukemia. Blood advances. 2024;8(12):3200-13.
84. Pollard JA, Guest E, Alonzo TA, Gerbing RB, Loken MR, Brodersen LE, et al. Gemtuzumab Ozogamicin Improves Event-Free Survival and Reduces Relapse in Pediatric KMT2A-Rearranged AML: Results From the Phase III Children's Oncology Group Trial AAML0531. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2021;39(28):3149-60.
85. Coenen EA, Zwaan CM, Reinhardt D, Harrison CJ, Haas OA, de Haas V, et al. Pediatric acute myeloid leukemia with t(8;16)(p11;p13), a distinct clinical and biological entity: a collaborative study by the International-Berlin-Frankfurt-Munster AML-study group. Blood. 2013;122(15):2704-13.
86. Noort S, Zimmermann M, Reinhardt D, Cuccuini W, Pigazzi M, Smith J, et al. Prognostic impact of t(16;21)(p11;q22) and t(16;21)(q24;q22) in pediatric AML: a retrospective study by the I-BFM Study Group. Blood. 2018;132(15):1584-92.
87. Hollink IH, van den Heuvel-Eibrink MM, Arentsen-Peters ST, Pratcorona M, Abbas S, Kuipers JE, et al. NUP98/NSD1 characterizes a novel poor prognostic group in acute myeloid leukemia with a distinct HOX gene expression pattern. Blood. 2011;118(13):3645-56.

88. Bisio V, Zampini M, Tregnago C, Manara E, Salsi V, Di Meglio A, et al. NUP98-fusion transcripts characterize different biological entities within acute myeloid leukemia: a report from the AIEOP-AML group. Leukemia. 2017;31(4):974-7.
89. Struski S, Lagarde S, Bories P, Puiseux C, Prade N, Cuccuini W, et al. NUP98 is rearranged in 3.8% of pediatric AML forming a clinical and molecular homogenous group with a poor prognosis. Leukemia. 2017;31(3):565-72.
90. de Rooij JD, Branstetter C, Ma J, Li Y, Walsh MP, Cheng J, et al. Pediatric non-Down syndrome acute megakaryoblastic leukemia is characterized by distinct genomic subsets with varying outcomes. Nat Genet. 2017;49(3):451-6.
91. Bertrums EJM, Smith JL, Harmon L, Ries RE, Wang YJ, Alonzo TA, et al. Comprehensive molecular and clinical characterization of NUP98 fusions in pediatric acute myeloid leukemia. Haematologica. 2023;108(8):2044-58.
92. Ostronoff F, Othus M, Gerbing RB, Loken MR, Raimondi SC, Hirsch BA, et al. NUP98/NSD1 and FLT3/ITD coexpression is more prevalent in younger AML patients and leads to induction failure: a COG and SWOG report. Blood. 2014;124(15):2400-7.
93. Heikamp EB, Henrich JA, Perner F, Wong EM, Hatton C, Wen Y, et al. The menin-MLL1 interaction is a molecular dependency in NUP98-rearranged AML. Blood. 2022;139(6):894-906.
94. Rasouli M, Blair H, Troester S, Szoltysek K, Cameron R, Ashtiani M, et al. The MLL-Menin Interaction is a Therapeutic Vulnerability in NUP98-rearranged AML. Hemasphere. 2023;7(8):e935.
95. Gruber TA, Larson Gedman A, Zhang J, Koss CS, Marada S, Ta HQ, et al. An Inv(16)(p13.3q24.3)-encoded CBFA2T3-GLIS2 fusion protein defines an aggressive subtype of pediatric acute megakaryoblastic leukemia. Cancer cell. 2012;22(5):683-97.
96. Masetti R, Pigazzi M, Togni M, Astolfi A, Indio V, Manara E, et al. CBFA2T3-GLIS2 fusion transcript is a novel common feature in pediatric, cytogenetically normal AML, not restricted to FAB M7 subtype. Blood. 2013;121(17):3469-72.
97. Smith JL, Ries RE, Hylkema T, Alonzo TA, Gerbing RB, Santaguida MT, et al. Comprehensive Transcriptome Profiling of Cryptic CBFA2T3-GLIS2 Fusion-Positive AML Defines Novel Therapeutic Options: A COG and TARGET Pediatric AML Study. Clinical cancer research : an official journal of the American Association for Cancer Research. 2020;26(3):726-37.

98. de Rooij JD, Masetti R, van den Heuvel-Eibrink MM, Cayuela JM, Trka J, Reinhardt D, et al. Recurrent abnormalities can be used for risk group stratification in pediatric AMKL: a retrospective intergroup study. Blood. 2016;127(26):3424-30.
99. Tang T, Le Q, Castro S, Pardo L, McKay CN, Perkins L, et al. Targeting FOLR1 in high-risk CBF2AT3-GLIS2 pediatric AML with STRO-002 FOLR1-antibody-drug conjugate. Blood advances. 2022;6(22):5933-7.
100. Bolouri H, Farrar JE, Triche T, Ries RE, Lim EL, Alonzo TA, et al. The molecular landscape of pediatric acute myeloid leukemia reveals recurrent structural alterations and age-specific mutational interactions. Nature Medicine. 2018;24(1):103-12.
101. Marceau-Renaut A, Duployez N, Ducourneau B, Labopin M, Petit A, Rousseau A, et al. Molecular Profiling Defines Distinct Prognostic Subgroups in Childhood AML: A Report From the French ELAM02 Study Group. Hemasphere. 2018;2(1):e31.
102. Grafone T, Palmisano M, Nicci C, Storti S. An overview on the role of FLT3-tyrosine kinase receptor in acute myeloid leukemia: biology and treatment. Oncol Rev. 2012;6(1):e8.
103. Tarlock K, Hansen ME, Hylkema T, Ries R, Farrar JE, Auvil JG, et al. Discovery and Functional Validation of Novel Pediatric Specific FLT3 Activating Mutations in Acute Myeloid Leukemia: Results from the COG/NCI Target Initiative. Blood. 2015;126(23):87-.
104. Dohner H, Wei AH, Appelbaum FR, Craddock C, DiNardo CD, Dombret H, et al. Diagnosis and Management of AML in Adults: 2022 ELN Recommendations from an International Expert Panel. Blood. 2022.
105. Tarlock K, Gerbing RB, Ries RE, Smith JL, Leonti A, Huang BJ, et al. Prognostic impact of cooccurring mutations in FLT3-ITD pediatric acute myeloid leukemia. Blood advances. 2024;8(9):2094-103.
106. Li S, Li N, Chen Y, Zheng Z, Guo Y. FLT3-TKD in the prognosis of patients with acute myeloid leukemia: A meta-analysis. Front Oncol. 2023;13:1086846.
107. Heuser M, Freeman SD, Ossenkoppele GJ, Buccisano F, Hourigan CS, Ngai LL, et al. 2021 Update on MRD in acute myeloid leukemia: a consensus document from the European LeukemiaNet MRD Working Party. Blood. 2021;138(26):2753-67.

108. Brown P, McIntyre E, Rau R, Meshinchi S, Lacayo N, Dahl G, et al. The incidence and clinical significance of nucleophosmin mutations in childhood AML. Blood. 2007;110(3):979-85.
109. Hollink IH, Zwaan CM, Zimmermann M, Arentsen-Peters TC, Pieters R, Cloos J, et al. Favorable prognostic impact of NPM1 gene mutations in childhood acute myeloid leukemia, with emphasis on cytogenetically normal AML. Leukemia. 2009;23(2):262-70.
110. Brunetti L, Gundry MC, Sorcini D, Guzman AG, Huang YH, Ramabadran R, et al. Mutant NPM1 Maintains the Leukemic State through HOX Expression. Cancer cell. 2018;34(3):499-512 e9.
111. Cuglievan B, Kantarjian H, Rubnitz JE, Cooper TM, Zwaan CM, Pollard JA, et al. Menin inhibitors in pediatric acute leukemia: a comprehensive review and recommendations to accelerate progress in collaboration with adult leukemia and the international community. Leukemia. 2024;38(10):2073-84.
112. Ho PA, Alonzo TA, Gerbing RB, Pollard J, Stirewalt DL, Hurwitz C, et al. Prevalence and prognostic implications of CEBPA mutations in pediatric acute myeloid leukemia (AML): a report from the Children's Oncology Group. Blood. 2009;113(26):6558-66.
113. Tarlock K, Lamble AJ, Wang YC, Gerbing RB, Ries RE, Loken MR, et al. CEBPA-bZip mutations are associated with favorable prognosis in de novo AML: a report from the Children's Oncology Group. Blood. 2021;138(13):1137-47.
114. Taube F, Georgi JA, Kramer M, Stasik S, Middeke JM, Rollig C, et al. CEBPA mutations in 4708 patients with acute myeloid leukemia: differential impact of bZIP and TAD mutations on outcome. Blood. 2022;139(1):87-103.
115. Tarlock K, Zhong S, He Y, Ries R, Severson E, Bailey M, et al. Distinct age-associated molecular profiles in acute myeloid leukemia defined by comprehensive clinical genomic profiling. Oncotarget. 2018;9(41):26417-30.
116. Stratmann S, Yones SA, Mayrhofer M, Norgren N, Skaftason A, Sun JT, et al. Genomic characterization of relapsed acute myeloid leukemia reveals novel putative therapeutic targets. Blood Advances. 2021;5(3):900-12.

117. Umeda M, Ma J, Huang BJ, Hagiwara K, Westover T, Abdelhamed S, et al. Integrated Genomic Analysis Identifies UBTF Tandem Duplications as a Recurrent Lesion in Pediatric Acute Myeloid Leukemia. Blood Cancer Discov. 2022;3(3):194-207.
118. McNeer NA, Philip J, Geiger H, Ries RE, Lavallée VP, Walsh M, et al. Genetic mechanisms of primary chemotherapy resistance in pediatric acute myeloid leukemia. Leukemia. 2019;33(8):1934-43.
119. Ma X, Liu Y, Liu Y, Alexandrov LB, Edmonson MN, Gawad C, et al. Pan-cancer genome and transcriptome analyses of 1,699 paediatric leukaemias and solid tumours. Nature. 2018;555(7696):371-6.
120. Barajas JM, Umeda M, Contreras L, Khanlari M, Westover T, Walsh MP, et al. UBTF tandem duplications in pediatric myelodysplastic syndrome and acute myeloid leukemia: implications for clinical screening and diagnosis. Haematologica. 2024;109(8):2459-68.
121. Khoury JD, Solary E, Abla O, Akkari Y, Alaggio R, Apperley JF, et al. The 5th edition of the World Health Organization Classification of Haematolymphoid Tumours: Myeloid and Histiocytic/Dendritic Neoplasms. Leukemia. 2022;36(7):1703-19.
122. Segerink WH, de Haas V, Kaspers GJL. Measurable residual disease in pediatric acute myeloid leukemia: a systematic review. Expert Rev Anticancer Ther. 2021;21(4):451-9.
123. Godley LA, DiNardo CD, Bolton K. Germline Predisposition in Hematologic Malignancies: Testing, Management, and Implications. Am Soc Clin Oncol Educ Book. 2024;44(3):e432218.
124. Creutzig U, Buchner T, Sauerland MC, Zimmermann M, Reinhardt D, Dohner H, et al. Significance of age in acute myeloid leukemia patients younger than 30 years: a common analysis of the pediatric trials AML-BFM 93/98 and the adult trials AMLCG 92/99 and AMLSG HD93/98A. Cancer. 2008;112(3):562-71.
125. Umeda M, Ma J, Westover T, Ni Y, Song G, Maciaszek JL, et al. A new genomic framework to categorize pediatric acute myeloid leukemia. Nat Genet. 2024;56(2):281-93.
126. Harrison CJ, Hills RK, Moorman AV, Grimwade DJ, Hann I, Webb DK, et al. Cytogenetics of childhood acute myeloid leukemia: United Kingdom Medical Research Council Treatment trials AML 10 and 12. J Clin Oncol. 2010;28(16):2674-81.

127. Hollink IHIM, Zwaan CM, Zimmermann M, Arentsen-Peters TCJM, Pieters R, Cloos J, et al. Favorable prognostic impact of NPM1 gene mutations in childhood acute myeloid leukemia, with emphasis on cytogenetically normal AML. Leukemia. 2009;23(2):262-70.
128. Kantarjian H, Kadia T, DiNardo C, Daver N, Borthakur G, Jabbour E, et al. Acute myeloid leukemia: current progress and future directions. Blood cancer journal. 2021;11(2):41.
129. Bhatt NS, Baassiri MJ, Liu W, Bhakta N, Chemaitilly W, Ehrhardt MJ, et al. Late outcomes in survivors of childhood acute myeloid leukemia: a report from the St. Jude Lifetime Cohort Study. Leukemia. 2021;35(8):2258-73.
130. Ganzel C, Lee JW, Fernandez HF, Paietta EM, Luger SM, Lazarus HM, et al. CNS involvement in AML at diagnosis is rare and does not affect response or survival: data from 11 ECOG-ACRIN trials. Blood advances. 2021;5(22):4560-8.
131. Kaspers GJ, Creutzig U. Pediatric acute myeloid leukemia: international progress and future directions. Leukemia. 2005;19(12):2025-9.
132. Kaspers GJ, Zimmermann M, Reinhardt D, Gibson BE, Tamminga RY, Aleinikova O, et al. Improved outcome in pediatric relapsed acute myeloid leukemia: results of a randomized trial on liposomal daunorubicin by the International BFM Study Group. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2013;31(5):599-607.
133. Pui CH, Schrappe M, Ribeiro RC, Niemeyer CM. Childhood and adolescent lymphoid and myeloid leukemia. Hematology Am Soc Hematol Educ Program. 2004:118-45.
134. Zwaan CM, Kolb EA, Reinhardt D, Abrahamsson J, Adachi S, Aplenc R, et al. Collaborative Efforts Driving Progress in Pediatric Acute Myeloid Leukemia. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2015;33(27):2949-62.
135. Gamis AS, Alonzo TA, Meshinchi S, Sung L, Gerbing RB, Raimondi SC, et al. Gemtuzumab ozogamicin in children and adolescents with de novo acute myeloid leukemia improves event-free survival by reducing relapse risk: results from the randomized phase III Children's Oncology Group trial AAML0531. Journal of clinical

oncology : official journal of the American Society of Clinical Oncology. 2014;32(27):3021-32.

136. Norsworthy KJ, Ko CW, Lee JE, Liu J, John CS, Przepiorka D, et al. FDA Approval Summary: Mylotarg for Treatment of Patients with Relapsed or Refractory CD33-Positive Acute Myeloid Leukemia. Oncologist. 2018;23(9):1103-8.

137. Ali S, Dunmore HM, Karres D, Hay JL, Salmonsson T, Gisselbrecht C, et al. The EMA Review of Mylotarg (Gemtuzumab Ozogamicin) for the Treatment of Acute Myeloid Leukemia. The Oncologist. 2019;24(5):e171-e9.

138. Perel Y, Auvrignon A, Leblanc T, Michel G, Reguerre Y, Vannier JP, et al. Treatment of childhood acute myeloblastic leukemia: dose intensification improves outcome and maintenance therapy is of no benefit--multicenter studies of the French LAME (Leucemie Aigue Myeloblastique Enfant) Cooperative Group. Leukemia. 2005;19(12):2082-9.

139. Pollard JA, Alonzo TA, Gerbing R, Brown P, Fox E, Choi J, et al. Sorafenib in Combination With Standard Chemotherapy for Children With High Allelic Ratio FLT3/ITD+ Acute Myeloid Leukemia: A Report From the Children's Oncology Group Protocol AAML1031. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2022;40(18):2023-35.

140. Huls G, Chitu DA, Havelange V, Jongen-Lavrencic M, van de Loosdrecht AA, Biemond BJ, et al. Azacitidine maintenance after intensive chemotherapy improves DFS in older AML patients. Blood. 2019;133(13):1457-64.

141. Roboz GJ, Ravandi F, Wei AH, Dombret H, Thol F, Voso MT, et al. Oral azacitidine prolongs survival of patients with AML in remission independently of measurable residual disease status. Blood. 2022;139(14):2145-55.

142. Burchert A, Bug G, Fritz LV, Finke J, Stelljes M, Rollig C, et al. Sorafenib Maintenance After Allogeneic Hematopoietic Stem Cell Transplantation for Acute Myeloid Leukemia With FLT3-Internal Tandem Duplication Mutation (SORMAIN). Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2020;38(26):2993-3002.

143. Cornelissen JJ, Blaise D. Hematopoietic stem cell transplantation for patients with AML in first complete remission. Blood. 2016;127(1):62-70.

144. Heinicke T, Krahl R, Kahl C, Cross M, Scholl S, Wolf HH, et al. Allogeneic hematopoietic stem cell transplantation improves long-term outcome for relapsed AML patients across all ages: results from two East German Study Group Hematology and Oncology (OSHO) trials. Ann Hematol. 2021;100(9):2387-98.
145. McDonald GB, Sandmaier BM, Mielcarek M, Sorror M, Pergam SA, Cheng GS, et al. Survival, Nonrelapse Mortality, and Relapse-Related Mortality After Allogeneic Hematopoietic Cell Transplantation: Comparing 2003-2007 Versus 2013-2017 Cohorts. Ann Intern Med. 2020;172(4):229-39.
146. Pollard JA, Loken M, Gerbing RB, Raimondi SC, Hirsch BA, Aplenc R, et al. CD33 Expression and Its Association With Gemtuzumab Ozogamicin Response: Results From the Randomized Phase III Children's Oncology Group Trial AAML0531. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2016;34(7):747-55.
147. Jones LM, Tarlock K, Cooper T. Targeted Therapy in Pediatric AML: An Evolving Landscape. Paediatr Drugs. 2021;23(5):485-97.
148. DiNardo CD, Jonas BA, Pullarkat V, Thirman MJ, Garcia JS, Wei AH, et al. Azacitidine and Venetoclax in Previously Untreated Acute Myeloid Leukemia. The New England journal of medicine. 2020;383(7):617-29.
149. Issa GC, Aldoss I, DiPersio J, Cuglievan B, Stone R, Arellano M, et al. The menin inhibitor revumenib in KMT2A-rearranged or NPM1-mutant leukaemia. Nature. 2023;615(7954):920-4.
150. Erba HP, Fathi AT, Issa GC, Altman JK, Montesinos P, Patnaik MM, et al. Update on a Phase 1/2 First-in-Human Study of the Menin-KMT2A (MLL) Inhibitor Ziftomenib (KO-539) in Patients with Relapsed or Refractory Acute Myeloid Leukemia. Blood. 2022;140(Supplement 1):153-6.
151. Feurstein S, Trottier AM, Estrada-Merly N, Pozsgai M, McNeely K, Drazer MW, et al. Germ line predisposition variants occur in myelodysplastic syndrome patients of all ages. Blood. 2022;140(24):2533-48.
152. Feurstein S, Churpek JE, Walsh T, Keel S, Hakkarainen M, Schroeder T, et al. Germline variants drive myelodysplastic syndrome in young adults. Leukemia. 2021;35(8):2439-44.

153. Sahoo SS, Kozyra EJ, Wlodarski MW. Germline predisposition in myeloid neoplasms: Unique genetic and clinical features of GATA2 deficiency and SAMD9/SAMD9L syndromes. Best Pract Res Clin Haematol. 2020;33(3):101197.
154. Arber DA, Orazi A, Hasserjian R, Thiele J, Borowitz MJ, Le Beau MM, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood. 2016;127(20):2391-405.
155. Trottier AM, Godley LA. Inherited predisposition to haematopoietic malignancies: overcoming barriers and exploring opportunities. British journal of haematology. 2021;194(4):663-76.
156. Trottier AM, Feurstein S, Godley LA. Germline predisposition to myeloid neoplasms: Characteristics and management of high versus variable penetrance disorders. Best Pract Res Clin Haematol. 2024;37(1):101537.
157. Homan CC, Drazer MW, Yu K, Lawrence DM, Feng J, Arriola-Martinez L, et al. Somatic mutational landscape of hereditary hematopoietic malignancies caused by germline variants in RUNX1, GATA2, and DDX41. Blood advances. 2023;7(20):6092-107.
158. Rio-Machin A, Vulliamy T, Hug N, Walne A, Tawana K, Cardoso S, et al. The complex genetic landscape of familial MDS and AML reveals pathogenic germline variants. Nature Communications. 2020;11(1).
159. Kottaridis PD, Gale RE, Frew ME, Harrison G, Langabeer SE, Belton AA, et al. The presence of a FLT3 internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials. Blood. 2001;98(6):1752-9.
160. Burnett AK, Hills RK, Green C, Jenkinson S, Koo K, Patel Y, et al. The impact on outcome of the addition of all-trans retinoic acid to intensive chemotherapy in younger patients with nonacute promyelocytic acute myeloid leukemia: overall results and results in genotypic subgroups defined by mutations in NPM1, FLT3, and CEBPA. Blood. 2010;115(5):948-56.
161. Kirwan M, Vulliamy T, Marrone A, Walne AJ, Beswick R, Hillmen P, et al. Defining the pathogenic role of telomerase mutations in myelodysplastic syndrome and acute myeloid leukemia. Hum Mutat. 2009;30(11):1567-73.

162. Pui CH, Carroll WL, Meshinchi S, Arceci RJ. Biology, risk stratification, and therapy of pediatric acute leukemias: an update. J Clin Oncol. 2011;29(5):551-65.
163. Gatta G, Botta L, Rossi S, Aareleid T, Bielska-Lasota M, Clavel J, et al. Childhood cancer survival in Europe 1999-2007: results of EUROCARE-5--a population-based study. Lancet Oncol. 2014;15(1):35-47.
164. Bolouri H, Farrar JE, Triche T, Jr., Ries RE, Lim EL, Alonzo TA, et al. The molecular landscape of pediatric acute myeloid leukemia reveals recurrent structural alterations and age-specific mutational interactions. Nat Med. 2018;24(1):103-12.
165. Radtke I, Mullighan CG, Ishii M, Su X, Cheng J, Ma J, et al. Genomic analysis reveals few genetic alterations in pediatric acute myeloid leukemia. Proceedings of the National Academy of Sciences of the United States of America. 2009;106(31):12944-9.
166. Farrar JE, Schuback HL, Ries RE, Wai D, Hampton OA, Trevino LR, et al. Genomic Profiling of Pediatric Acute Myeloid Leukemia Reveals a Changing Mutational Landscape from Disease Diagnosis to Relapse. Cancer research. 2016;76(8):2197-205.
167. Shiba N, Yoshida K, Shiraishi Y, Okuno Y, Yamato G, Hara Y, et al. Whole-exome sequencing reveals the spectrum of gene mutations and the clonal evolution patterns in paediatric acute myeloid leukaemia. British journal of haematology. 2016;175(3):476-89.
168. Liu T, Rao J, Hu W, Cui B, Cai J, Liu Y, et al. Distinct genomic landscape of Chinese pediatric acute myeloid leukemia impacts clinical risk classification. Nat Commun. 2022;13(1):1640.
169. Grimwade D, Hills RK, Moorman AV, Walker H, Chatters S, Goldstone AH, et al. Refinement of cytogenetic classification in acute myeloid leukemia: determination of prognostic significance of rare recurring chromosomal abnormalities among 5876 younger adult patients treated in the United Kingdom Medical Research Council trials. Blood. 2010;116(3):354-65.
170. Tyner JW, Tognon CE, Bottomly D, Wilmot B, Kurtz SE, Savage SL, et al. Functional genomic landscape of acute myeloid leukaemia. Nature. 2018;562(7728):526-31.
171. Cancer Genome Atlas Research N, Ley TJ, Miller C, Ding L, Raphael BJ, Mungall AJ, et al. Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. N Engl J Med. 2013;368(22):2059-74.

172. Olsson L, Zettermark S, Biloglav A, Castor A, Behrendtz M, Forestier E, et al. The genetic landscape of paediatric de novo acute myeloid leukaemia as defined by single nucleotide polymorphism array and exon sequencing of 100 candidate genes. Br J Haematol. 2016;174(2):292-301.
173. Tarlock K, Alonzo TA, Wang YC, Gerbing RB, Ries R, Loken MR, et al. Functional Properties of KIT Mutations Are Associated with Differential Clinical Outcomes and Response to Targeted Therapeutics in CBF Acute Myeloid Leukemia. Clin Cancer Res. 2019;25(16):5038-48.
174. de Rooij JD, van den Heuvel-Eibrink MM, Hermkens MC, Verboon LJ, Arentsen-Peters ST, Fornerod M, et al. BCOR and BCORL1 mutations in pediatric acute myeloid leukemia. Haematologica. 2015;100(5):e194-5.
175. Lindsley RC, Mar BG, Mazzola E, Grauman PV, Shareef S, Allen SL, et al. Acute myeloid leukemia ontogeny is defined by distinct somatic mutations. Blood. 2015;125(9):1367-76.
176. Terada K, Yamaguchi H, Ueki T, Usuki K, Kobayashi Y, Tajika K, et al. Usefulness of BCOR gene mutation as a prognostic factor in acute myeloid leukemia with intermediate cytogenetic prognosis. Gene Chromosome Canc. 2018;57(8):401-8.
177. Grossmann V, Tiacci E, Holmes AB, Kohlmann A, Martelli MP, Kern W, et al. Whole-exome sequencing identifies somatic mutations of BCOR in acute myeloid leukemia with normal karyotype. Blood. 2011;118(23):6153-63.
178. Aly M, Ramdzan ZM, Nagata Y, Balasubramanian SK, Hosono N, Makishima H, et al. Distinct clinical and biological implications of CUX1 in myeloid neoplasms. Blood Advances. 2019;3(14):2164-78.
179. McNerney ME, Brown CD, Wang XY, Bartom ET, Karmakar S, Bandlamudi C, et al. CUX1 is a haploinsufficient tumor suppressor gene on chromosome 7 frequently inactivated in acute myeloid leukemia. Blood. 2013;121(6):975-83.
180. Wong CC, Martincorena I, Rust AG, Rashid M, Alifrangis C, Alexandrov LB, et al. Inactivating CUX1 mutations promote tumorigenesis. Nat Genet. 2014;46(1):33-+.
181. Sood R, Hansen NF, Donovan FX, Carrington B, Bucci D, Maskeri B, et al. Somatic mutational landscape of AML with inv(16) or t(8;21) identifies patterns of clonal evolution in relapse leukemia. Leukemia. 2016;30(2):501-4.

182. de Rooij JDE, van den Heuvel-Eibrink MM, van de Rijdt NKAM, Verboon LJ, de Haas V, Trka J, et al. PHF6 mutations in paediatric acute myeloid leukaemia. British Journal of Haematology. 2016;175(5):967-71.
183. Reinhardt D, Antoniou E, Waack K. Pediatric Acute Myeloid Leukemia-Past, Present, and Future. Journal of Clinical Medicine. 2022;11(3).
184. Eisfeld AK, Kohlschmidt J, Mims A, Nicolet D, Walker CJ, Blachly JS, et al. Additional gene mutations may refine the 2017 European LeukemiaNet classification in adult patients with de novo acute myeloid leukemia aged <60 years. Leukemia. 2020;34(12):3215-27.
185. Gardin C, Pautas C, Fournier E, Itzykson R, Lemasle E, Bourhis JH, et al. Added prognostic value of secondary AML-like gene mutations in ELN intermediate-risk older AML: ALFA-1200 study results. Blood Adv. 2020;4(9):1942-9.
186. Herold T, Rothenberg-Thurley M, Grunwald VV, Janke H, Goerlich D, Sauerland MC, et al. Validation and refinement of the revised 2017 European LeukemiaNet genetic risk stratification of acute myeloid leukemia. Leukemia. 2020;34(12):3161-72.
187. Tazi Y, Ossa JA, Zhou YY, Bernard E, Thomas I, Gilkes A, et al. Unified classification and risk-stratification in Acute Myeloid Leukemia. Nature Communications. 2022;13(1).
188. Hollink IHIM, van den Heuvel-Eibrink MM, Arentsen-Peters STCJM, Zimmermann M, Peeters JK, Valk PJM, et al. Characterization of CEBPA mutations and promoter hypermethylation in pediatric acute myeloid leukemia. Haematol-Hematol J. 2011;96(3):384-92.
189. Matsuo H, Kajihara M, Tomizawa D, Watanabe T, Saito AM, Fujimoto J, et al. Prognostic implications of CEBPA mutations in pediatric acute myeloid leukemia: a report from the Japanese Pediatric Leukemia/Lymphoma Study Group. Blood Cancer Journal. 2014;4.
190. Theodoris CV, Mourkioti F, Huang Y, Ranade SS, Liu L, Blau HM, et al. Long telomeres protect against age-dependent cardiac disease caused by NOTCH1 haploinsufficiency. J Clin Invest. 2017;127(5):1683-8.
191. Lomelí H, Castillo-Castellanos F. Notch signaling and the emergence of hematopoietic stem cells. Dev Dynam. 2020;249(11):1302-17.

## IX. BIBLIOGRAPHY OF THE CANDIDATE'S PUBLICATIONS

### Publications related to the dissertation


1. **Krizsan S**, Peterffy B, Egyed B, Nagy T, Sebestyen E, Hegyi LL, Jakab Z, Erdelyi DJ, Muller J, Peter G, Csanadi K, Kallay K, Krivan G, Barna G, Bedics G, Haltrich I, Ottoffy G, Csernus K, Vojcek A, Tiszlavicz LG, Gabor KM, Kelemen A, Hauser P, Gaal Z, Szegedi I, Ujfalusi A, Kajtar B, Kiss C, Matolcsy A, Timar B, Kovacs G, Alpar D, Bodor C. Next-Generation Sequencing-Based Genomic Profiling of Children with Acute Myeloid Leukemia. J Mol Diagn. 2023;25(8):555-68. **IF: 3.4**

2. Armes H, Rio-Machin A, **Krizsan S**, Bodor C, Kaya F, Bewicke-Copley F, Alnajar J, Walne A, Peterffy B, Tummala H, Rouault-Pierre K, Dokal I, Vulliamy T, Fitzgibbon J. Acquired somatic variants in inherited myeloid malignancies. Leukemia. 2022;36(5):1377-81. **IF: 11.4**

3. **Krizsán S**, Dénes Z, Gángó A, Gerecs B, Demeter J., Nagy Z , Tárkányi I, Masszi T, Farkas P, Masszi A, Szombath G, Benedek S, Várkonyi J, Horváth L, Nagy Z, Radványi G, Takács I, Hamed A, Lázár Z, Süveges E, Kárpáti Á, Plander M, Szendrei T, Pál K, Gurzó M, Jakucs J, Egyed M, Bödör C. Analysis of recurrent genetic abnormalities in acute myeloid leukemia in the context of novel targeted therapies. Hematológia–Transzfuziológia 2019; 52(2): 96-105. (Hungarian)

4. **Krizsán S**, Hegyi L, Masszi T, Bödör C. Recent advances in molecular diagnostics and targeted therapy of acute myeloid leukemia. Orvosképzés. 2021;96(3): 567-80. (Hungarian)

5. Alpár D, Egyed B, **Krizsán S**, Erdélyi D, Bödör C, Kovács, G. State-of-the-art molecular diagnostics and treatment of pediatric acute leukemia. Orvosképzés. 2021;96(3):507–20. (Hungarian)

**Other publications**

1. Kiss R, Papp G, **Krizsán S**, Kotmayer L, Gángó A, Nagy N, Bátai B, Mátrai Z, Bödör C, Alpár D. Screening for genomic copy number alterations in chronic lymphocytic leukemia using multiplex ligation-dependent probe amplification. Hematológia–Transzfuziológia 2018;51(1):31-40. (Hungarian)

2. Nagy Á, Ősz Á, Budczies J, **Krizsán S**, Szombath G, Demeter J, Bödör C, Győrffy B. Elevated HOX gene expression in acute myeloid leukemia is associated with *NPM1* mutations and poor survival. J Adv Res. 2019;20:105-16. **IF: 6.992**

3. Kiss R, Gángó A, Benard-Slagter A, Egyed B, Haltrich I, Hegyi L, de Groot K, Király PA, **Krizsán S**, Kajtár B, Pikó H, Pajor L, Vojcek Á, Matolcsy A, Kovács G, Szuhai K, Savola S, Bödör C, Alpár D. Comprehensive profiling of disease-relevant copy number aberrations for advanced clinical diagnostics of pediatric acute lymphoblastic leukemia. Mod Pathol. 2020;33(5):812-824. **IF: 7.842**

4. Raska A, **Krizsán S,** Nagy Z, Demeter J, Bödör C, Tárkányi I. Evaluation of acute myeloid leukemia patients treated between 2007 and 2017 at the 1st Department of Internal Medicine, Semmelweis University. Hematológia–Transzfuziológia. 2019;52(4):215-24. (Hungarian)

5. Ifj. Nagy B, Gángó A, Rejtő L, **Krizsán S**, Ujfalusi A, Antal-Szalmás P. Laboratory analysis and prognostic values of *CEBPA* gene mutations in acute myeloid leukemia. Hematológia–Transzfuziológia. 2019;52(1):11-7. (Hungarian)

6. Gángó A, Alpár D, Galik B, Marosvári D, Kiss R, Fésüs V, Aczél D, Eyüpoglu E, Nagy N, Nagy Á, **Krizsán S**, Reiniger L, Farkas P, Kozma A, Ádám E, Tasnády S, Réti M, Matolcsy A, Gyenesei A, Mátrai Z, Bödör C. Dissection of subclonal evolution by temporal mutation profiling in chronic lymphocytic

leukemia patients treated with ibrutinib. Int J Cancer. 2020;146(1):85-93. **IF: 7.396**

7. Batai B, **Krizsan S**, Gango A, Hegyi L, Csoka M, Erdelyi DJ, Csomor J, Kallay K, Bodor C. Juvenile myelomonocytic leukaemia presentation after preceding juvenile xanthogranuloma harbouring an identical somatic PTPN11 mutation. Pediatr Blood Cancer. 2020;67(9):e28368.

8. Lin WY, Fordham SE, Hungate E, Sunter NJ, Elstob C, Xu Y, Park C, Quante A, Strauch K, Gieger C, Skol A, Rahman T, Sucheston-Campbell L, Wang J, Hahn T, Clay-Gilmour AI, Jones GL, Marr HJ, Jackson GH, Menne T, Collin M, Ivey A, Hills RK, Burnett AK, Russell NH, Fitzgibbon J, Larson RA, Le Beau MM, Stock W, Heidenreich O, Alharbi A, Allsup DJ, Houlston RS, Norden J, Dickinson AM, Douglas E, Lendrem C, Daly AK, Palm L, Piechocki K, Jeffries S, Bornhauser M, Rollig C, Altmann H, Ruhnke L, Kunadt D, Wagenfuhr L, Cordell HJ, Darlay R, Andersen MK, Fontana MC, Martinelli G, Marconi G, Sanz MA, Cervera J, Gomez-Segui I, Cluzeau T, Moreilhon C, Raynaud S, Sill H, Voso MT, Lo-Coco F, Dombret H, Cheok M, Preudhomme C, Gale RE, Linch D, Gaal-Wesinger J, Masszi A, Nowak D, Hofmann WK, Gilkes A, Porkka K, Milosevic Feenstra JD, Kralovics R, Grimwade D, Meggendorfer M, Haferlach T, **Krizsan S**, Bodor C, Stolzel F, Onel K, Allan JM. Genome-wide association study identifies susceptibility loci for acute myeloid leukemia. Nat Commun. 2021;12(1):6233. **IF: 17.694**

9. Casado P, Rio-Machin A, Miettinen JJ, Bewicke-Copley F, Rouault-Pierre K, **Krizsan S**, Parsons A, Rajeeve V, Miraki-Moud F, Taussig DC, Bodor C, Gribben J, Heckman C, Fitzgibbon J, Cutillas PR. Integrative phosphoproteomics defines two biologically distinct groups of *KMT2A* rearranged acute myeloid leukaemia with different drug response phenotypes. Signal Transduct Target Ther. 2023;8(1):80. **IF: 40.8**

10. Bedics G, Egyed B, Kotmayer L, Benard-Slagter A, de Groot K, Beko A, Hegyi LL, Batai B, **Krizsan S**, Krivan G, Erdelyi DJ, Muller J, Haltrich I, Kajtar B, Pajor

L, Vojcek A, Ottoffy G, Ujfalusi A, Szegedi I, Tiszlavicz LG, Bartyik K, Csanadi K, Peter G, Simon R, Hauser P, Kelemen A, Sebestyen E, Jakab Z, Matolcsy A, Kiss C, Kovacs G, Savola S, Bodor C, Alpar D. PersonALL: a genetic scoring guide for personalized risk assessment in pediatric B-cell precursor acute lymphoblastic leukemia. Br J Cancer. 2023;129(3):455-65. **IF: 6.4**

11. Péterffy B, Nádasi TJ, Krizsán S, Horváth A, Márk Á, Barna G, Timár B, Almási L, Müller J, Csanádi K, Rakonczai A, Nagy Z, Kállay K, Kertész G, Kriván G, Csóka M, Sebestyén A, Semsei ÁF, Kovács GT, Erdélyi DJ, Bödör C, Egyed B, Alpár D. Digital PCR-based quantification of miR-181a in the cerebrospinal fluid aids patient stratification in pediatric acute lymphoblastic leukemia. Sci Rep. 2024;14(1):28556. **IF: 3.8**

12. Péterffy B, Krizsán S, Egyed B, Bedics G, Benard-Slagter A, Palit S, Erdélyi DJ, Müller J, Nagy T, Hegyi LL, Bekő A, Kenéz LA, Jakab Z, Péter G, Zombori M, Csanádi K, Ottóffy G, Csernus K, Vojcek Á, Tiszlavicz LG, Gábor KM, Kelemen Á, Hauser P, Kállay K, Kertész G, Gaál Z, Szegedi I, Barna G, Márk Á, Haltrich I, Hevessy Z, Ujfalusi A, Kajtár B, Timár B, Kiss C, Kriván G, Matolcsy A, Savola S, Kovács G, Bödör C, Alpár D. Molecular profiling reveals novel gene fusions and genetic markers for refined patient stratification in pediatric acute lymphoblastic leukemia. Mod Pathol. 2025;38(6):100741. Epub ahead of print. **IF: 7.1**

## X. ACKNOWLEDGEMENT

I would like to express my sincere gratitude to András Matolcsy, MD, PhD, DSc, Head of the Department of Pathology and Experimental Cancer Research at Semmelweis University, for the opportunity to pursue PhD studies at this Institute.

I owe great thanks to my supervisors, Csaba Bödör, PhD, DSc, and Donát Alpár, PhD, for their kind support and unwavering help and for guiding me through my PhD studies.

I wish to thank Richárd Kiss, MD, PhD and Ambrus Gángó, MD, PhD, for taking their time to teach me fundamental laboratory techniques at the beginning of my research.

I want to express my gratitude to Noémi Nagy, PhD for her guidance and thoughtfulness over the years we worked together.

I owe special thanks to Borbála Péterffy, MD, for working together in pediatric leukemia research over the years. Her dedication to science and work ethics is impeccable, and working with her in the routine diagnostics of pediatric leukemic patients was a pleasure.

I wish to thank all the colleagues and members of the HCEMM-SE Molecular Oncohematology Research Group for creating an inspiring work environment every day.

I am also grateful to every Department of Pathology and Experimental Cancer Research employee for their support and helpfulness in every situation.

In addition, I would like to thank the Hungarian Pediatric Oncology Network members who contributed to my research with clinical data and with whom, in collaboration, we launched the Hungarian Pediatric Leukemia Molecular Profiling Program.

I wish to thank Cecilia Laczik for her kind help regarding the administrative part of my PhD program.

Finally, I greatly thank my husband, daughter, and foster mother for their love and constant support throughout my studies. Without their help, none of this would have been possible.